Expression und funktionelle Implikationen des Corticosteroid-bindenden Globulins/CBG

Dissertation

zur Erlangung des akademischen Grades
doctor medicinea (Dr. med.)

vorgelegt dem Rat der Medizinischen Fakultät
der Friedrich-Schiller-Universität Jena

von Benjamin Möpert
geboren am 08.02.1980 in Brandenburg/Havel

Gutachter

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Abkürzungsverzeichnis

ACTH	−	adrenocorticotropes Hormon
cAMP	−	Zyklisches Adenosinmonophosphat
CBG	−	Corticosteroid-bindendes Globulin
CORT	−	Cortisol resp. Corticosteron
CRH	−	Corticotropin-Releasing Hormon
ELISA	−	enzyme linked immunosorbant assay
GR	−	Glucocorticoid-Rezeptor
HP	−	Hypophyse
HPA-Achse	−	Hypothalamo-Hypohysen-Nebennierenrinden-Achse
HT	−	Hypothalamus
kDa	−	Kilodalton
mRNA	−	Messenger Ribonucleinsäure
NNRI	−	Nebennierenrindeninsuffizienz
NSO	−	Nucleus supraopticus
NVP	−	Nucleus paraventricularis
OT	−	Oxytocin
RT-PCR	−	Reverse Transkriptase-Polymerase-Kettenreaktion
SHBG	−	Sex hormone binding globulin
VP	−	Vasopressin
ZNS	−	Zentrales Nervensystem

Inhaltsverzeichnis

1 Zusammenfassung

Das Corticosteroid-bindende Globulin (CBG), ein spezifisches Transportprotein für adrenale Steroide im Blutplasma mit hoher Affinität zu Glucocorticoiden (CORT), konnte bei vielen Vertebraten nachgewiesen werden. Dabei zeigte sich, dass es neben der Leber, seinem Hauptproduktionsort, auch in diversen anderen Geweben vorhanden ist und zum Teil exprimiert wird. Neuere Studien weisen darauf hin, dass die Funktion des CBG weit über die eines reinen Steroidtransporters hinausgeht.

Hauptaufgabe dieser Arbeit war es, mittels kombinierter Immunhistochemie die morphologische Verteilung des CBG im zentralen Nervensystem (ZNS) der Ratte darzustellen, es auf die Koexistenz mit den Neuropeptiden Oxytocin (OT) und Vasopressin (VP) zu prüfen und eventuelle funktionelle Konsequenzen zu diskutieren. Weiterhin wurde untersucht, ob die Plasmakonzentration von CBG beim Menschen tageszeitlichen Schwankungen unterliegt.

Wir konnten zeigen, dass CBG im ZNS der Ratte mit unterschiedlicher Lokalisation und Verteilung vorkommt. Hauptlokalisationsorte sind Neurone der magnozellulären hypothalamischen Kerngebiete und deren Projektionen. Darüber hinaus findet es sich in Projektionen und Axonvarikositäten der Emenentia mediana, des Hypophysenhinterlappens und des Zwischenhirns sowie in Zellen des Hypophysenvorderlappens und einzelnen Ependymzellen. Das Verteilungsmuster impliziert unterschiedliche Funktionen für das Protein.

Durch eine intraventrikuläre Colchizinapplikation zeigte sich, als Zeichen einer Akkumulation, eine deutlich gesteigerte Färbeintensität für CBG, ohne dass sich dabei das Verteilungsmuster veränderte. Gemeinsam mit der spezifischen Färbung von Projektionen und Axonvarikositäten deutet dies auf einen axonalen Transport, eine vesikuläre Speicherung sowie auf eine eventuelle neuro-hormonartige Ausschüttung von CBG hin.

Mittels In Situ-Hybridisierung auf Semidünnschnitten von Rattengehirnen konnten wir zeigen, dass CBG in Zellen des Hypothalamus (HT) exprimiert wird. Dieses Ergebnis konnte später von Jirikowski et al. (2007) durch RT-PCR von homogenisiertem Hypothalamusmaterial bestätigt werden.

Mit kombinierter Immunoperoxidasefärbung und Immunofluoreszenz konnten wir die Koexistenz von CBG und OT, und in geringerem Maße von CBG und VP in magnozellulären Neuronen des Hypothalamus nachweisen.

Die Verteilung des CBG im ZNS der Ratte, seine intrinsische Expression, der axonale Transport sowie die Kolokalisation mit den Neuropeptiden OT und VP weisen darauf hin, dass CBG eine Reihe komplexer Funktionen ausübt. Dazu gehören die Steuerung der Fraktion von freiem und somit biologisch aktiven CORT innerhalb und außerhalb von Zellen im ZNS, eine mögliche Vermittlung und Regulation der Feedbackmechanismen des CORT auf HT- und Hypophysen- (HP) Ebene sowie ein möglicher Einfluss auf die stressmodulierenden Eigenschaften von OT und VP. Auch eine Funktion als hypophyseotroper Faktor ist denkbar. Darüber hinaus könnte es die schon früher beschriebenen, schnellen nicht-genomischen Wirkungen von CORT an Zellen mittels eines Membranrezeptors für CBG vermitteln.

Zusammengenommen ist eine Rolle für CBG innerhalb der komplexen Regulationsmechanismen der neuroendrokrinen Stressantwort wahrscheinlich.

Aufgabe des zweiten Teils dieser Arbeit war es zu prüfen, ob die CBG-Konzentration im Plasma des Menschen tageszeitlichen Schwankungen unterliegt.

Die CBG Plasmakonzentrations-Analyse mittels ELISA von je 13 Proben (entnommen zwischen 8:00 Uhr und 17:30 Uhr) von 20 gesunden Probanden zeigte einen signifikanten Anstieg von zirkulierendem CBG zwischen 12:30 Uhr und 13:00 Uhr. Diese Konzentrations-erhöhung könnte den verminderten Anstieg der Konzentration von freiem CORT im Plasma am frühen Nachmittag, verglichen mit der CORT-Gesamtkonzentration, erklären. Das bedeutet, dass CBG Einfluss auf die freie CORT-Konzentration des Blutes hat. Im Umkehrschluss kann durch Messung der Konzentration von CBG und des Gesamt-CORT die Konzentration des freien CORT im Plasma errechnet werden. Eine mögliche Anwendung wäre eine bessere Überwachung der tatsächlichen Hormonsituation bei schweren Erkrankungen, wie etwa dem septischen Schock, durch Ermittlung der freien und damit aktiven CORT-Konzentration. Dies könnte helfen eine genauere Indikation zur Substitutionstherapie mit Hydrocortison zu stellen.

2 Einleitung

2.1 Die HPA-Achse und die Wirkungen der Glucocorticoide

Unter den zahlreichen physiologischen Stress-Systemen nimmt die Hypothalamus-Hypophysen-Nebennierenrinden-Achse (HPA-Achse) eine Sonderstellung ein. Bei der HPA-Achse handelt es sich um ein dreigliedriges Hormonsystem, welches aus dem hypothalamischen Peptidhormon Corticotropin-Releasing Hormone (CRH), dem hypophysären adrenocorticotropen Hormone (ACTH) sowie dem Nebennierenrinden-Steroid Cortisol resp. Corticosteron (CORT) besteht. Die drei sezernierten Hormone bilden mehrere negative Rückkopplungsschleifen, um auf allen drei Gewebsebenen eine optimale Regulation der Produktion und Sekretion dieser Botenstoffe zu gewährleisten.

Glucocorticoide (CORT) spielen eine entscheidende Rolle in der Aufrechterhaltung des homöostatischen Gleichgewichtes und haben vielfältigste Effekte auf Zellen und Organsysteme im gesamten Organismus. Sie steuern die Reaktionen eines Lebewesens auf Stress, regulieren dabei den Stoffwechsel und haben Einfluss auf die Immunlage. Auf Grund ihrer lipophilen Eigenschaften können Glucocorticoide die Blut-Hirn Schranke überwinden. Im zentralen Nervensystem (ZNS) steuern sie unter anderem neuroendokrine Funktionen wie das Stressverhalten, das Sexualverhalten und wurden mit Aspekten des Lernens sowie der Entstehung von Emotionen in Verbindung gebracht (Tasker 2006). Inwieweit CORT weitere Wirkungen im ZNS ausübt und welche genauen Wirkmechanismen dabei bestehen ist weitgehend ungeklärt. Bekannt ist jedoch, dass ein Überschuss an CORT im ZNS, insbesondere im Hippocampus, Zellen schädigt und zerstört (Sapolsky 1996). Bemerkenswert ist außerdem, dass Patienten mit schwerer Depression häufig eine Dysregulation der HPA-Achse und damit einen erhöhten CORT-Spiegel im Serum aufweisen (Caroll et al. 2007). Ob hier ein Zusammenhang besteht ist bislang unklar.

2.2 Das CBG – Eigenschaften, Lokalisation und mögliche biologische Rolle

Das Corticosteroid-bindende Globulin (CBG) ist ein Glycoprotein mit einem Molekülgewicht von 46kDa und gehört strukturell zur Familie der Serinprotease-Inhibitoren. Als spezifisches Transportprotein für Steroide im Blutplasma wird es auch als Transcortin bezeichnet. Es besitzt eine einzelne Bindungsstelle für Steroide, weist eine hohe Affinität zu Glucocorticoiden auf und bindet mehr als 90% des zirkulierenden CORT im Blutplasma.

Neben der Leber als Hauptproduktionsort (Hammond et al. 1991) konnte die Expression von CBG in verschiedenen Geweben unterschiedlicher Spezies nachgewiesen werden. Dazu gehören Lunge (Seralini et al. 1990), Nieren (Scrocchi et al. 1993), Plazenta (Misao et al. 1999), Ovarien (Misao et al. 1997), Hypophysenvorderlappen (de Kloet et al. 1984), Liquor (Predine et al. 1984) und weißes Fettgewebe (del Mar Grasa et al. 2001). 2006 konnten Herbert et al. das funktionsverwandte Steroidbindungsprotein SHBG im Hypothalamus (HT) der Ratte nachweisen und zeigen, dass es mit Oxytocin (OT) kolokalisiert und axonal transportiert wird.

Der möglichen Funktion des CBG liegen zwei elementar verschiedene Theorien zugrunde. So geht eine dieser Theorien davon aus, dass nur der freie ungebundene Anteil des Serum-CORT biologisch aktiv ist („free hormone hypothesis“[Mendel 1989]). In diesem Kontext wären die Funktionen des CBG dann erhöhte CORT-Konzentrationen abzupuffern, Hormon in Zielgeweben bereitzustellen, die renale Clearancerate zu regulieren und somit die Fraktion freien und damit biologisch aktivem CORT auf lokaler und systemischer Ebene zu beeinflussen (Hammond et al. 1991; Breuner und Orchinik 2002; Dey und Roychowdhury 2003). Kuhn (1988) und Nakhla et al. (1988) zeigten, dass CBG aktiv von Zellen internalisiert werden kann. Dieser Mechanismus könnte dazu beitragen die intrazelluläre Konzentration von CORT in stärkerem Maße zu steigern als durch Diffusion allein (Breuner und Orchinik 2002).

Die zweite Theorie über die Funktion des CBG nimmt an, dass CBG eine entscheidende Rolle bei der Vermittlung der schnellen, nicht-genomischen Effekte von CORT an Zellmembranen spielt. So fand man eine rezeptorähnliche Bindungsstruktur auf Zellmembranen unterschiedlicher Gewebe, welche spezifisch CBG banden. Die Bindung von CORT-beladenem CBG initiierte einen G-Protein vermittelten Prozess, welcher über die

Induktion der Adenylatzyklase die cAMP Konzentration in der Zelle steigerte (Hryb et al. 1986, Singer et al. 1988, Strel'chyonok und Avvakumov 1991, Maitra et al. 1993, Nakhla et al. 1988).

Je nachdem welche Theorie man der Interpretation neuer Erkenntnisse zugrunde legt entstehen unterschiedliche funktionelle Konsequenzen. In dieser Arbeit wurde versucht die Ergebnisse im Kontext beider Theorien zu diskutieren.

2.3 Oxytocin und Vasopressin im zentralen Nervensystem

Es ist bekannt, dass Glucocorticoide dosisabhängig die Sekretion von OT und Vasopressin (VP) beeinflussen. OT und VP sind ihrerseits an den komplexen Mechanismen der Stressantwort beteiligt. VP wird überwiegend im HT von magnozellulären Neuronen des Nucleus supraopticus (NSO) und des Nucleus paraventricularis (NPV) synthetisiert (Choy und Watkins 1977; Rhodes et al. 1981). Neben seiner Funktion als Regulator des Plasmavolumens, der Osmolarität des Blutes und des Blutdruckes wird VP zusammen mit dem Releasingfaktor CRH in der Emenentia mediana in den Hypothalamo-hypophysären Kreislauf ausgeschüttet (de Goeij et al. 1991). Über V1b Rezeptoren auf adenohypophysären ACTH-Zellen verstärkt VP die CRH-Wirkung und hat so Einfluss auf die Produktion von Stresshormonen in der Nebennierenrinde (Aguilera und Rabadan-Diehl 2000; Volpi et al. 2004). Auch OT wird in den magnozellulären Neuronen des NPV und NSO gebildet (Choy und Watkins 1977; Rhodes et al. 1981). Es wird für die Steuerung von Brutpflegeverhalten verantwortlich gemacht (Pedersen et al. 1982) und hat Effekte auf Lernen und Erinnerung (Fehm-Wolfsdorf et al. 1984). Panksepp et al. (1997) und Windle et al. (1997) demonstrierten, dass OT darüber hinaus eine zentral gesteuerte stress- und angstlösende Wirkung zu haben scheint. Trotzdem die Produktionsorte von OT und VP zu klassischen Glucocorticoid-Zielgeweben zählen weisen nur wenige der dort befindlichen Zellen nukleäre Glucocorticoid-Rezeptoren (GR) auf (Jirikowski et al. 1993).

2.4 Das CBG im Blutplasma

Betrachtet man nochmals die einzelnen Stationen der HPA-Achse so wird deutlich, dass die Modulation der Antwort eines Organismus auf Stress auf unterschiedlichen Ebenen erfolgen kann. Es ist bekannt dass die Ausschüttung von CORT impulsweise erfolgt und dabei einer zirkadianen Rhythmik unterliegt. 90% des Gesamt-CORT im Plasma sind an das entsprechende Haupt-Transportprotein CBG gebunden. Geht man davon aus, dass nur der Anteil des frei zirkulierenden CORT biologisch aktiv ist, ergibt sich für das CBG eine neue Bedeutung. Es könnte möglicherweise über seine Rolle als reines Transportprotein hinaus, an der Regulation des freien Anteils von CORT mitbeteiligt sein (Breuner und Orchinik 2002). Um die Bedeutung von CBG in der Modulation der peripheren Stressantwort besser verstehen zu können ist es nötig zu ermitteln, ob auch die Plasmakonzentration des CBG tageszeitlichen Schwankungen unterliegt. Bei Ratten fand man bereits eine gewisse Tageszeit-abhängige Veränderung der CBG-Konzentration im Serum (Hsu und Kuhn 1988). Im zweiten Teil dieser Arbeit sollte dies am Menschen untersucht werden.

Diese Arbeit entstand unter der Leitung meines Betreuers Prof. Dr. Gustav F. Jirikowski und mit Hilfe von John G. Lewis vom Steroid Lab. der Canterbury Health Laboratories in Christchurch Neuseeland, der die im Rahmen meines Stipendiums durchgeführten Untersuchungen zur Variation der Plasmakonzentration von CBG leitete. Sie bettet sich in den Versuch ihrer Arbeitsgruppen ein, die komplexe Natur der Steroidbindungsproteine zu ergründen und zu verstehen.

3 Ziele der Arbeit

Aufgabe der vorliegenden Arbeit war es, mittels immunhistochemischer und biochemischer Verfahren die Verteilung und Expression von CBG im zentralen Nervensystem der Ratte zu ermitteln. Dabei wurde spezielles Augenmerk auf eine mögliche Kolokalisation mit den Neuropeptiden Oxytocin und Vasopressin gelegt und dies mit Doppelimmunofluoreszenz-Verfahren überprüft. Eventuelle funktionelle Konsequenzen sollten diskutiert werden.

Es sollte außerdem der Frage nachgegangen werden, ob die Blutplasmakonzentration des CBG beim Menschen tageszeitlichen Schwankungen unterliegt und welchen Einfluss diese Schwankungen auf den CORT-Spiegel im Blut haben. Für diesen Teil der Arbeit führte ich funktionelle Untersuchungen im Steroid Lab. der Canterbury Health Laboratories in Christchurch, Neuseeland mittels Immunoassays für CBG am Menschen durch.

Die Ergebnisse der Arbeit wurden in begutachteten internationalen Fachzeitschriften veröffentlicht. Diese Publikationen bilden das Kernstück der vorliegenden kumulativen Dissertation.

4 Publizierte Originalarbeiten

4.1

Möpert B, Herbert Z, Caldwell JD, Jirikowski GF. 2006.

Expression of Corticosteron Binding Globulin CBG in the rat hypothalamus.

Horm Metab Res, 38(4): 246-252

Expression of Corticosterone-binding Globulin in the Rat Hypothalamus

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Original

Abstract

We observed coexistence of corticosteroid-binding globulin (CBG) with vasopressin (VP) and oxytocin (OT) in magnocellular neurons in rat hypothalamus by combined immunoperoxidase staining and immunofluorescence. A portion of the supraoptic and of the paraventricular neurons showed double immunostaining of CBG with either VP or with OT. CBG staining was intensified by pretreating animals with colchicine to block axonal transport. CBG was also observed in widespread axonal projections throughout the lateral hypothalamus, the median eminence and the posterior pituitary lobe. Single ependymal cells and some of the endocrine cells in the anterior lobe contained specific CBG immunoreactivity. *In situ* hybridization of semithin sections with a synthetic oligonucleotide probe to CBG mRNA provided staining of magnocellular hypothalamic neurons, but not ependymal cells or anterior lobe cells. Western blots of CBG extracted by affinity chromatography from hypothalamus homogenates showed a band at approximately 50 kDa. Our observations indicate the intrinsic expression of CBG in peptidergic hypothalamus neurons in rat. The multiple locations of CBG-expressing neurons indicate multiple functional properties, probably exceeding the role of a mere steroid transporter. CBG is likely to be subject to axonal transport and secretion in a neuropeptide-like fashion, perhaps involved in neuroendocrine regulation, which may include stress responses.

Key words
Corticosterone · steroid · vasopressin · oxytocin · posterior lobe · hypothalamus · axonal transport

Introduction

Corticosteroid-binding globulin (CBG) is a 46 kDa glycoprotein that has been shown to be expressed in mouse liver and kidney [1] and in rat adipose tissue [2]. CBG was also observed in endocrine cells of the anterior pituitary lobe using immunocytochemistry and in brain tissue by biochemical methods [3, 4].

CBG has a single steroid-binding site with high affinity to glucocorticoids such as cortisol in humans and corticosterone in rat [4 – 8]. Adrenal steroids may control hypothalamic neuroendocrine pathways. Due to their lipophilic nature, glucocorticoids are capable of crossing the blood-brain barrier and are known to block oxytocin (OT) secretion while enhancing vasopressin (VP) secretion, both in a dose-dependent manner, as part of the endocrine chain of stress responses [9 – 16]. VP is also known to modulate stress response such as by enhancing the actions of corticotropin releasing factor CRF on anterior lobe corticotrophs [51]. Panksepp et al. [17] showed that OT injections into domestic chicks decreased distress calls during separation from the mother hen, indicating that the stress of that situation was reduced after OT treatment. Windle et al. [18] demonstrated that OT treatment reduced corticosterone release stimulated by stress, suggesting that OT is anxiolytic. Hypothalamic magnocellular nuclei such as the paraventricular nucleus (PVN) are functional glucocorticoid targets [19 – 22]. Nevertheless, nuclear glucocorti-

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Received 27 October 2005 · **Accepted after revision** 8 February 2006

Bibliography
Horm Metab Res 2006; 38: 246–252 © Georg Thieme Verlag KG Stuttgart · New York ·
DOI 10.1055/s-2006-925344 · ISSN 0018-5043

coid receptors are only present in scattered magnocellular neurons [13].

Similar to other steroid-binding proteins such as sex hormone-binding globulin (SHBG) and the closely related androgen-binding protein (ABP), CBG has long been thought to function solely as a carrier for plasma steroids to their target tissues. More recently, membrane associated receptors have been postulated for SHBG [23 – 26] and CBG [27], thus possibly accounting for known rapid non-genomic actions by their respective steroids.

Recently, the intrinsic expression of ABP and of SHBG was observed in the rat hypothalamo-neurohypophyseal system [28], in part co-localized with OT [29] or with vasopressin [30]. Hypothalamic SHBG may be subject to axoplasmic transport.

In this study, we used immunocytochemistry and *in situ* hybridization to assess the expression of CBG in the hypothalamus and the pituitary of intact and of colchicine pretreated rats. Double immunostaining with either VP or OT was employed to characterize the peptidergic nature of CBG-positive neurons further. Western blots of affinity-purified hypothalamus extracts were immunostained for CBG for further verification.

Materials and Methods

Intact adult male rats, BW 300 g, (n = 3) were briefly anesthetized with ether to receive single unilateral injections of colchicine (5 µl of a 5% solution in isotonic sterile saline) into the lateral ventricle, according to an earlier described protocol [31]. Six male rats of the same size were left untreated with colchicine. After a survival time of 24 h, all animals were killed by prolonged ether anesthesia prior to cardiac perfusion with 4% paraformaldehyde in PBS (0.1 M sodium phosphate buffer pH 7.4 containing 0.9% NaCl). After fixation, brains were removed and postfixed overnight in the same fixative. Two of the uninjected animals were perfused only with isotonic saline for the biochemical studies. Hypothalami of these brains were rapidly dissected and frozen in liquid propane for biochemical analysis.

The three colchicine treated and 2 of the untreated brains were sectioned after postfixation on a vibratome (Lancer) into 100 µm serial frontal sections for immunocytochemistry.

Small tissue blocks containing the paraventricular nucleus were dissected from two of the formaldehyde-fixed untreated brains. These tissue blocks and all pituitaries were dehydrated through ascending ethanol series and propylene oxide, followed by embedding in EPON resin. Series of 1 µm semithin sections were cut from EPON blocks on a Reichert Ultracut microtome and collected on microscopic slides.

Immunocytochemistry

Vibratome sections of colchicine-treated animals and of controls were washed in PBS containing 0.5% Triton X 100 (PBS-TX), 3 × 60 min at RT, followed by incubation with anti CBG (polyclonal rabbit antiserum, Affiland Inc.) diluted in PBS 1 : 250 overnight at 4 °C. After that, sections were washed in PBS-TX and incubated with biotinylated anti-rabbit IgG (Vector lab Inc.) diluted to 1 : 100 in PBS-TX for 1 h at RT. Immunoprecipitates were visualized with streptavidin peroxidase complex (Vector lab. Inc.), diluted to 1 : 100 in PBS for 1 h at RT, followed by development with diaminobenzidine (DAB) and H_2O_2 in PBS for 5 min.

For double immunostaining, CBG-stained sections were briefly rinsed in 0.1 M sodium citrate buffer pH 4.0 to remove immune complexes from the previous reaction. Sections were then washed in PBS-TX and incubated with either rabbit anti-OT (AB 911, Chemicon, Temecula, USA) or rabbit anti-VP (AB 937, Chemicon, Temecula, USA). The specificity of these antibodies is well-established. Antibodies were diluted 1 : 1000 in PBS; incubation time was overnight at 4 °C. Sections were then washed in PBS and incubated for 2 h in CY3-labeled anti rabbit IgG Fab-fragment (Sigma), diluted at 1 : 100 in PBS. In some settings, OT or VP staining was performed with DAB followed by CBG staining with CY3. After washing in PBS, sections were affixed onto microscopic slides and mounted with N-propyl-gallate-glycerine.

Semithin sections were briefly treated with a 10% sodium methoxide solution for removal of resin followed by rehydration and immersion in PBS. Immunostaining for CBG was performed as described above. Immunocytochemical controls were performed with normal rabbit serum instead of the specific antiserum.

In situ hybridization

A synthetic oligonucleotide probe (Dr. Birch-Hirschfeld, Dept. of Virology, FSU, Jena, Germany) complementary to CBG encoding mRNA was labeled by 3' tailing with 5'bromo-2'desoxyuridine (BrdU) as described earlier [29]. The sequence of the antisense probe was 5'ATG ACC TTG GAG ATG TGC TC according to the NIH database. The respective sense probe was used for control hybridizations. Probes were labeled by tailing with 5'bromo-2'desoxyuridine (BrdU, Sigma) and terminal transferase (tailing kit, Boehringer Mannheim).

Epoxy resin was removed from semithin hypothalamus sections as described above by omitting the rehydration step. Sections were briefly air-dried and immediately hybridized with 20 pM of the respective sense or antisense probe dissolved in hybridization buffer Omnibuff (Jenabiotech, Jena). Hybridization was performed for 4 h at 37 °C in a humid chamber. Sections were then washed in PBS, hybridization product was visualized with a monoclonal antibody to BrdU (Progen, Heidelberg), diluted 1 : 400 in PBS, followed by anti-mouse IgG, 1 : 200 in PBS (1 h at RT), mouse-PAP complex, 1 : 200 (1 hat RT) and DAB – H_2O_2 (all reagents obtained from Sigma).

Immunostained and hybridized sections were examined with an Olympus BX 50 photo microscope with interference contrast illumination. Filter combination WU and epifluorescence was used for detection of CY3 staining. A digital camera DP 10 and Olympus DP soft computer program was used for documenting histochemical images.

Affinity chromatography

Frozen hypothalami from intact rats were rapidly thawed in lysis buffer (0.1 M PBS containing 2 nM $MgCl_2$, 5 mM EDTA, 0.1 mM PMSF, 1% CHAPS (3-[(3-Cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate), 50 mM leupeptin, 0.02% β-mercapto-etha-

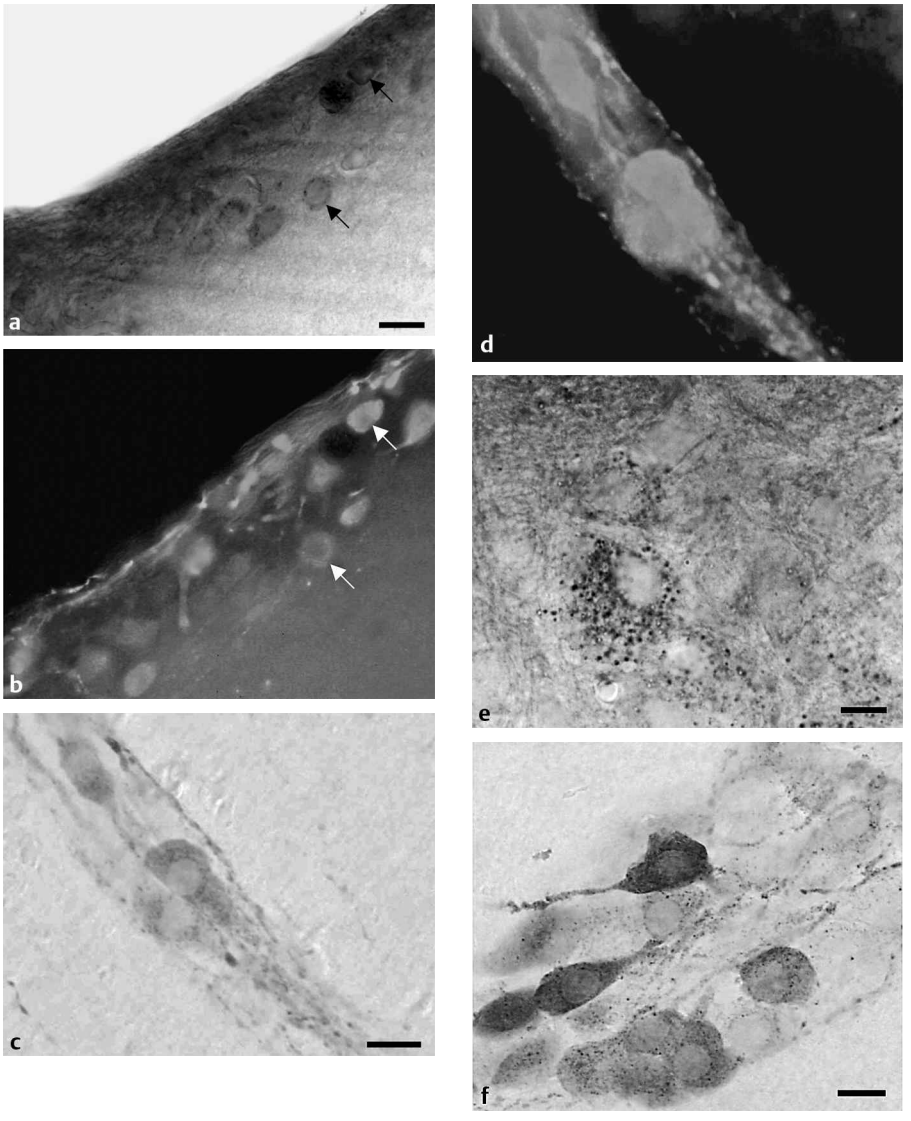

Fig. **1** **a, b** Vibratome section of the SON of an intact rat immunostained for CBG (**a**) reveals weak granulated reaction product in the perinuclear cytoplasm of single magnocellular neurons. Immunofluorescence of this section with CY3 for OT (**b**) shows a partial coexistence of both antigens (arrows). Scale bar = 20 µm. **c, d** Vibratome scection of the PVN double stained for CBG (**c**) and VP (**b**) shows co-localization of both antigens in single neurons. Scale bar = 10 µm. **e** Vibratome section of the PVN: Immunoperoxidase staining of magnocellular perikarya in untreated rats is mostly confined to granules in the perinuclear cytoplasm and in neuronal processes. Scale bar = 5 µm. **f** Vibratome section of the PVN from a colchizine treated rat immunostained for CBG shows intense cytoplasmic staining of magnocellular neurons and of their processes. Scale bar = 10µm.

nol) and homogenized mechanically, followed by sonication. Samples were centrifuged for 30 min at 10 000 rpm at 2 °C. The supernatant was applied in aliquots of 200 µl each to a protein A sepharose column (Sigma), which had been coated with the Affiland CBG antibody at a volume of 1 ml. Binding was performed overnight at 4 °C. Then columns were extensively washed with PBS until dot blots of the eluate proved to be protein free as determined by Coomassie Blue 250 (BioRad) staining. CBG bound to the column was eluted with 1 ml 0.1 M sodium acetate buffer at pH 4.2. Eluates were collected and dialyzed against distilled water overnight. Columns were reconditioned with PBS and again incubated with 200 µl of hypothalamus or pituitary homogenate, respectively, for the next extraction run. Samples were dried in a Speed Vac concentrator.

Western Blot

Dried protein samples were resuspended in sterile distilled water and separated by SDS electrophoresis in a polyacrylamide gradient gel. After separation, gels were blotted onto nitrocellulose membranes.

Membranes were cross-linked by 10 min treatment with UV light (Transilluminator, Biometra). After that, blots were rinsed with PBS-TX and incubated with rabbit anti-CBG (Fitzgerald Industries Int. Inc. Concord, MA USA) diluted to 1 : 500 in PBS overnight at 4 °C. After washing in PBS-TX, blots were incubated with anti rabbit IgG (1 : 200 in PBS-TX, 1 h at RT) and streptavidin peroxidase complex (1 : 200 in PBS-TX, 1 h at RT). Diaminobenzidine and H_2O_2 were used to visualize immunoprecipitates.

Results

CBG immunostaining was observed in a fraction of the neurons of the supraoptic and the paraventricular nuclei as well as the periventricular nucleus of the hypothalamus. Single CBG positive perikarya also appeared in the lateral hypothalamus, the bed nucleus of the stria terminalis, the preoptic region and in close apposition to the parvocellular, (CBG-negative) neurons of the suprachiasmatic nucleus. Cells were almost exclusively magnocellular with a mean perikaryal diameter of approximately 15 µm. Immunoreactivity was, in general, relatively weak in untreated rats. Staining was restricted to a granular reaction product (Fig. **1 e**) in the perinuclear cytoplasm, while the nuclei were devoid of specific staining. Numerous immunostained neuronal processes with varicosities appeared in the lateral hypothala-

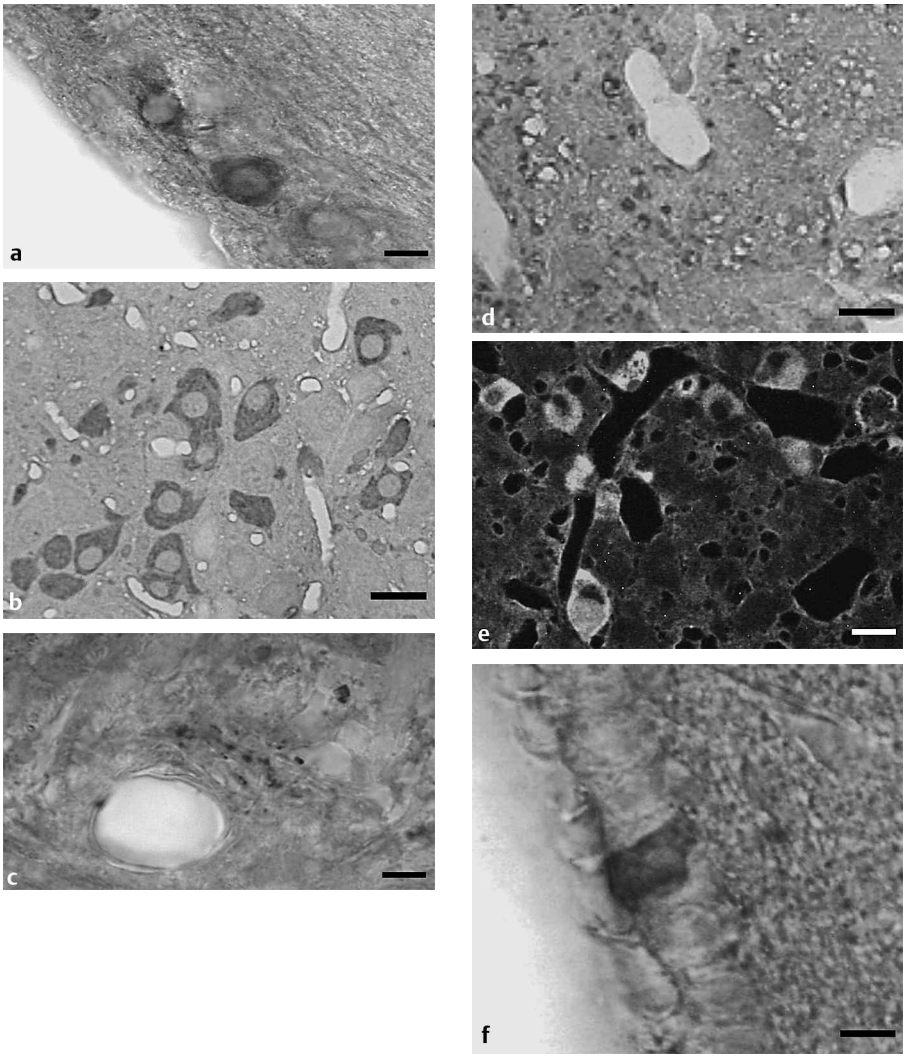

Fig. **2** **a** Immunostaining for CBG is observed in single magnocellular perikarya in the periventricular nucleus in close apposition to the ependymal layer. Scale bar = 10 µm. **b** Semithin section of the PVN of an untreated rat: *In situ* hybridization with a BrdU-labeled oligonucleotide probe to CBG-encoding mRNA results in BrdU immunoreactivity in a portion of the magnocellular neurons. Scale bar = 15 µm. **c** Immunoperoxidase staining for CBG in a semithin section of the median eminence shows immunoreactive varicosities in the internal zone in close apposition to a blood vessel. Scale bar = 5 µm. **d** Semithin section of the posterior pituitary lobe contains numerous CBG positive Herring bodies. Scale bar = 10 µm. **e** Semithin section of the anterior pituitary lobe shows CY3 immunofluorescence for CBG in a portion of the endocrine cells. Scale bar = 5µm. **f** Vibratome section: Single ependymal cells are immunostained for CBG. Scale bar = 5 µm.

mus, suprachiasmatic nucleus, bed nucleus of the stria terminalis, preoptic region and arcuate nucleus. Single magnocellular neurons in the periventricular nucleus showed clear cytoplasmic CBG immunoreactivity (Fig. **2 a**). Specific CBG staining also appeared in single ependymal cells (Fig. **2 f**). Immunocytochemical control sections remained unstained. Staining intensity of CBG immunoreactive neurons was drastically increased in animals that had been treated with colchicine 24 h prior to sacrifice (Fig. **1 f**). However, the overall topography of CBG-positive cells was not affected by colchicine treatment.

VP or OT immunofluorescence staining of vibratome sections that had been prestained with CBG revealed a partial coexistence of CBG with the classical magnocellular peptides (Fig. **1 a, b**). While only single magnocellular neurons in the PVN and SON appeared to be vasopressinergic (Fig. 1 **c, d**), about one third of the OT cells showed CBG immunoreactivity. Comprehensive cell counts were not performed in this morphological study.

Immunostaining of semithin hypothalamus sections revealed CBG reactivity in axonal varicosities in both the internal and the external layer of the median eminence and in Herring bodies of the posterior pituitary lobe (Fig. **2 d**). We observed CBG-positive axon terminals in close apposition to blood vessels in the internal layer of the ME (Fig. **2 c**).

Single endocrine cells in the anterior pituitary lobe were clearly labeled for CBG as determined by immunofluorescence of semithin sections (Fig. **2 e**). Immunocytochemical controls were devoid of stained reaction product.

Immunostaining of semithin sections for BrdU following *in situ* hybridization with the BrdU-labeled oligonucleotide probe showed specific cytoplasmic immunoreactivity in some magnocellular neurons in the SON, PVN (Fig. **2 b**), PEV and in some perivascular neurons. This staining was not visible in the control incubations that had been hybridized with the sense probe instead of the specific antisense probe. Hybridization signal was not seen in the ependymal layer lining the third ventricle.

Western blots of the affinity purified hypothalamus and pituitary homogenates showed one clearly stained band at a molecular weight of approximately 50 kDa for the hypothalamus. A similar band could be observed in preparations of the anterior pituitary as well as in the posterior lobe (Fig. **3**).

Discussion

This is the first demonstration of CBG immunoreactivity at the cellular level in the rat hypothalamus. Our observations with *in situ* hybridization suggest an intrinsic expression of CBG in rat

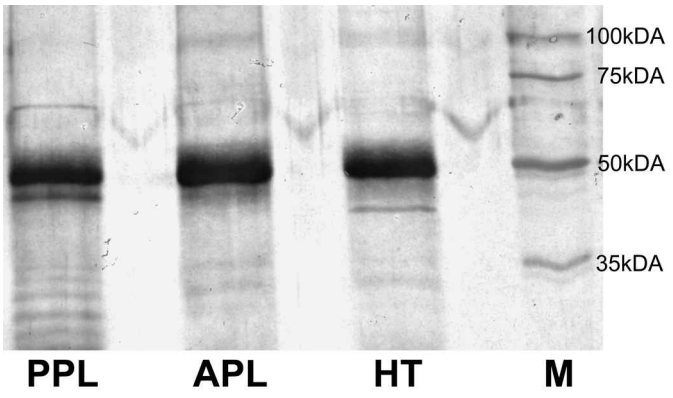

Fig. **3** Western Blot of affinity purified extracts from PPL: posterior pituitary lobe, APL: anterior pituitary lobe, HT: hypothalamus; immunostained for CBG: A distinct band is visible at approximately 50 kDa. M: molecular weight marker.

hypothalamic neurons, rather than the accumulation of serum CBG by these cells. Due to its size, the CBG glycoprotein is unlikely to cross the blood-brain barrier. Localization of CBG in axons and axonal varicosities in the lateral hypothalamus, the median eminence and the posterior pituitary indicates neurohormone-like transport and storage. We found CBG in magnocellular neurons within the PVN, the SON, and associated nuclei, partially co-localized with the posterior lobe hormones VP and OT. Numerous CBG immunostained neuronal processes appeared in the lateral hypothalamus, suprachiasmatic nucleus, bed nucleus of the stria terminalis, preoptic region and arcuate nucleus. Oxytocinergic and vasopressinergic neurons have been shown to project to the mesencephalon, the brain stem and the preoptic region [32]. Since CBG is present in some of these neurons, the respective projections are likely to exist also for CBG. Previous immunocytochemical experiments failed to find this steroid-binding globulin in the hypothalamus [3]. These studies, however, did not pre-treat animals with colchicine, which we found greatly increases CBG immunoreactivity. Colchicine treatment, which is known to inhibit axonal transport by disrupting microtubules, resulted in a perikaryal accumulation of CBG. This further supports the idea that CBG is subject to high secretory turnover under normal conditions. Whether CBG can be released from nerve terminals has not been shown so far. For SHBG, we demonstrated localization in dense-core secretory vesicles by immunoelectron microscopy, which supports this hypothesis. Such experiments are currently performed for hypothalamic CBG.

Co-localization of the steroid-binding protein and the classical neuropeptides OT and AVP is likely of functional significance. While CBG in the periphery is important for control of the hypothalamo-pituitary-adrenal (HPA) axis, it's possible role in brain has not been examined. However, it is reasonable to assert that CBG in brain is also involved in stress responses. VP is not only involved in control of ACTH release [33], but has also been associated with stress. Engelmann et al. [34] demonstrated that the forced swim test released VP from numerous brain areas while "social defeat" stress-released OT, suggesting that both VP and OT are involved in responses to stress, but that they may respond differently under different conditions. A primary function of OT is its release during parturition [35–37], which is also a very stressful event.

Both OT and VP and now CBG are found in the PVN, which is a critical brain area in control of the HPA axis. The PVN is the primary CRH-producing area of the brain [19,21], and is therefore important for controlling adrenocorticotropin release. Glucocorticoids are known to exert feedback control back on the brain via glucocorticoid receptors [19,38,39], but only few of the magnocelular hypothalamic perikarya are glucocorticoid targets [40]. Swanson and Simmons [21] found that corticosterone infusions into the brain activated CRH expression in the dorsal PVN indicating a responsiveness of the CRH production to brain glucocorticoid levels. Some of the parvocellular CRH neurons in the PVN have glucocorticoid receptors [22]. CBG in identical or in nearby neurons may be important in delivering glucocorticoids to CRH neurons and their intracellular glucocorticoid receptors.

We found CBG immunoreactivity in a distinct portion of ependymal cells lining the third ventricle. Breuner and Orchinik [41] demonstrated CBG immunoreactivity in cells lining the cerebral ventricles, which they attributed to uptake of CBG from the CSF. Our *in situ* hybridization experiments failed to find CBG encoding mRNA in ependymal cells of the third ventricle, which would corroborate this assertion. The CSF has been shown to contain CBG in humans [42–43].

CBG immunoreactivity was also evidenced in the internal layer of the median eminence, from where it may gain access to the portal circulation of the anterior pituitary lobe, along with classical releasing factors. CBG immunostaining was observed in numerous endocrine cells of the anterior lobe. Similar immunocytochemical observations, along with the respective mRNA, were made before [3], suggesting an intrinsic expression of CBG in a portion of the endocrine cells in the anterior pituitary lobe. Although we did not perform co-localization experiments with the anterior lobe peptides, the high number of stained cells, their morphology and their distribution indicate that CBG immunoreactivity is not confined to corticotrophs as previously suggested [3], but could also be found in some of the other endocrine cells.

CBG immunoreactivity was also found in a portion of Herring bodies in the posterior pituitary lobe. This allows for the assumption that CBG, at least here, is processed for release. This agrees with our previous observation that SHBG is localized in secretory vesicles in Herring bodies along with OT [29]. CBG may also be co-localized with either OT or VP in Herring bodies of the posterior pituitary.

Our western blots suggest that CBG extracted from hypothalamus, anterior lobe and from the posterior lobe stains at a molecular weight of approximately 50 kDa, which matches the known molecular weight of 46 kDa for CBG. This parallels our previous observations for SHBG, which showed with mass spectrometry that hypothalamic and systemic SHBG are identical molecules.

Our laboratory has some experience in examining two other steroid-binding globulins, SHBG and ABP. We have found that SHBG is made in the brain [29], where its production and release appear to be controlled by steroid hormone status [29,44]. We have also seen that central infusions of SHBG alter female sexual behavior [45,46] in a manner that suggests that SHBG plays a

role in mediating steroid activation of the brain. We have suggested that there are membrane-associated receptors for SHBG in the brain [47,48]. Strel'chnyok and Avvakumov [49] have described a binding site for CBG on the cell membrane. Nakhla et al. [50] have taken this idea further to suggest that CBG stimulation of its membrane-associated receptor activates intracellular adenylate-cyclase/cAMP second messenger systems. Both of these studies were performed on peripheral tissues or cells. It is unknown so far whether there is a CBG membrane receptor in the brain. Although the possible functions of CBG in brain have not been explored, the co-localization of CBG with the stress hormones OT and VP and its known involvement in the HPA axis suggest a role in control of stress responses. It is possible that membrane-associated receptors for CBG exist to mediate such central effects.

Clearly, the different locations of the corticosteroid-binding globulin in the rat brain indicate various different functions: In addition to being a factor in the hypothalamo-neurohypophyseal system, it may be a central neuroactive substance and a protein of anterior lobe endocrine cells. Most likely, the properties of central CBG exceed the function of a mere transporter for serum glucocorticoids.

Acknowledgements

Supported by NIH grant R21 MH 069 810 – 01A1 to JDC and GFJ.

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JirikowskiGF, Pusch L, Möpert B, Herbert Z, Caldwell JD. 2007.

Expression of corticosteroid binding globulin in the rat central nervous system.

J Chem Neuroanat, 34(1-2): 22-28

Available online at www.sciencedirect.com
ScienceDirect
Journal of Chemical Neuroanatomy 34 (2007) 22–28
Journal of CHEMICAL NEUROANATOMY
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Expression of corticosteroid binding globulin in the rat central nervous system

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Received 6 February 2007; received in revised form 27 March 2007; accepted 27 March 2007
Available online 31 March 2007

Abstract

Immunoreactivity for corticosteroid binding globulin was observed in the hypothalamus of intact male rats in the magnocellular nuclei and in single neurons in the periventricular nucleus and the lateral hypothalamus. The suprachiasmatic and the arcuate nuclei contained parvocellular neurons with specific immunoreactivity. Extensive networks of immunopositive fibers were observed in the lateral hypothalamus, the preoptic region, the bed nucleus of the stria terminalis and along the third ventricle. Immunostained axons often exhibited varicosities. The internal and the external layer of the median eminence showed numerous bundles of immunostained axons. Herring bodies in the posterior pituitary lobe contained specific immunoreactivity while pituicytes remained unstained. A portion of the Purkinje cells in the cerebellum and mossy fibers in the cerebellar granular layer stained for corticosteroid binding globulin. Some of the pyramidal cells in the hippocampus were corticosteroid binding globulin positive. Immunostained fibers occurred in the mesencephalon in the periaqueductal grey and in the medulla oblongata. A small fraction of the ependymal cells was also stained. In the spinal cord we observed specific immunoreactivity in a portion of the neurons in the dorsal horn. With polymerase chain reaction we confirmed the presence of the respective transcripts in the different brain regions.

The multiple locations of corticosteroid binding globulin throughout the central nervous system suggest multiple functional properties, including neuroendocrine and neurohumoral functions.

Keywords: Corticosteroid; Hypothalamus; Hippocampus; Hypophysial tract; Spinal cord; Localization

1. Introduction

In a recent study, we showed that corticosteroid binding globulin (CBG), a 46 kDa glycoprotein, is present in peptidergic hypothalamus neurons (Möpert et al., 2006). Pretreatment with colchicine increased CBG immunoreactivity, suggesting that the steroid binding protein is subject to axonal transport along with the classical neurohypophysial hormones (Möpert et al., 2006). In situ hybridization revealed the presence of CBG encoding mRNA in magnocellular hypothalamus neurons indicating CBG production in the hypothalamus. With immunocytochemical double staining we observed a partial colocalization with the antidiuretic posterior lobe hormone vasopressin (VP) and with the labor-inducing and milk-ejecting nonapeptide oxytocin (OT). Both neuropeptides as well as in the control of stress-related behaviours (Jezova et al., 1995).

CBG has been observed with immunocytochemistry in some of the endocrine cells of the anterior pituitary lobe and with biochemical methods in brain tissue (De Moor et al., 1962; De Kloet et al., 1984). Due to their lipophilic nature, steroid hormones are capable of crossing the blood brain barrier and stress-related behavioural changes are clearly modulated by adrenal steroids. However many of the glucocorticoid responsive brain areas in the hypothalamus or the limbic system contain only scattered neurons that express classical nuclear glucocorticoid receptors (GCR). Oxytocinergic perikarya are mostly devoid of GCR (Jirikowski et al., 1993). GCR knockout mice show no altered stress response (Barden, 1994). CBG has been suggested to be involved in known non-genomic actions of adrenal steroids and the existence of a membrane

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doi:10.1016/j.jchemneu.2007.03.007

associated CBG receptor has been proposed (Nakhla et al., 1988; Krupenko et al., 1991; Strel'chyonok and Avvakumov, 1991).

Since CBG and CBG encoding mRNA is found in various hypothalamic and extrahypothalamic areas, we addressed the question of the distribution of CBG immunoreactivity in the rat central nervous system. Here we investigated intact untreated rats, to circumvent non-specific colchicine effects on the neuroendocrine stress response. RT PCR of mRNA extracts, obtained from homogenates of the various brain regions was performed to get an idea about actual expression of CBG.

2. Materials and methods

Intact adult male rats, BW ca. 300 g ($n = 6$) were killed by extended ether anaesthesia, prior to cardiac perfusion with 4% paraformaldehyde in PBS (0.1 M sodium phosphate buffer pH 7.4 containing 0.9% NaCl). After fixation brains, pituitaries, and portions of the cervical spinal cord were removed and postfixed over night in the same fixative. Brains were sectioned on a vibratome (Lancer) into 100 μm serial frontal sections, to be processed for immunocytochemistry. Small tissue blocks of the pituitaries or cross-sections of the spinal cord were dehydrated through ascending ethanol series and acetonitril (Fluka), followed by embedding in EPON resin (Merck). After polymerization at 55 °C, Series of 1 μm semithin sections were cut on a Reichert Ultracut microtome and collected onto microscopic slides.

Additional matching animals ($n = 6$) were killed as described above and perfused with isotonic saline for the biochemical studies. Microdissected brain regions included the hypothalamic nuclei, hippocampus, cerebral cortex, cerebellum, the cervical portion of the spinal cord and pituitaries. Tissues were rapidly dissected and frozen in liquid nitrogen for RNA isolation. Special care was taken to separate the posterior pituitary lobe from the intermediate and the anterior lobe in these preparations.

2.1. Immunocytochemistry

Vibratome sections were washed in PBS containing 0.5% Triton X-100 (PBS-TX), three times for 60 min each at RT, followed by incubation with anti-CBG (polyclonal rabbit antiserum, Affiland Inc.) diluted in PBS 1:250, over night at 4 °C. Thereafter sections were washed in PBS-TX and incubated with biotinylated anti-rabbit IgG (Vector Lab. Inc.) diluted 1:100 in PBS-TX for 1 h at RT. Immunoprecipitates were visualized with streptavidin-peroxidase complex (Vector Lab. Inc.), diluted 1:100 in PBS for 1 h at RT, followed by development with diaminobenzidine (DAB) and H_2O_2 in PBS for 5 min. After washing in PBS, sections were affixed onto microscopic slides and mounted with *N*-propyl-gallate-glycerol.

Semithin sections (1 μm) of pituitaries and of the spinal cord were treated with a 10% sodium methoxide solution for removal of resin, followed by rehydration and immersion in PBS. Immunostaining for CBG was carried out as described above. Immunocytochemical controls were performed with normal rabbit serum instead of the specific antiserum.

Immunostained sections were examined with an Olympus BX 50 photo microscope and interference contrast illumination. A digital camera DP 10 and Olympus DP soft computer program was used for documentation of histochemical images. The stereotaxic atlas by Paxinos and Watson (1986) was used for reference of anatomical structures.

2.2. RNA extraction

RNA isolation was performed according to the Qiagen RNeasy protocol. Briefly, we extracted 0.2–5 μg of total RNA per sample. The resulting total RNA from each sample was eluted in 40 μl DEPC water and quantified spectrophotometrically at 260 nm. The RNA quality was also checked by 1.5% agarose gel electrophoresis and staining with 1 μg/ml ethidium bromide.

2.3. RT-PCR

A two-step RT-PCR method was used to detect CBG expression. 0.3 μg total RNAs were transcribed into cDNA using a mix for random priming and dT_{24VN} and SuperScript II reverse transcriptase (Invitrogen Life Technologies). Total RNA was combined with the mix of random priming and dT_{24VN} (50 pmol of each) and preheated to 70 °C for 8 min for denaturation of secondary structures. The mixture was cooled rapidly on ice and then the reaction mix containing 10 U of SuperScript II reverse transcriptase (Invitrogen Life Technologies) was added. Incubation was performed at 42 °C for 90 min and then stopped by increasing the temperature to 95 °C for 5 min. cDNA stock was stored at −20 °C. The yield of cDNA was measured according to the PCR signal generated from the internal standard house-keeping gene beta-actin.

For each gene to be assayed, intron-spanning primers were designed using the publicly available genomic coding sequences, obtained through NCBI ID (http://www.ncbi.nlm.nih.gov/entrez), (http://genome.ucsc.edu/cgi-bin/hgGateway) and the public domain primer design software Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

The primer sequences were: CBG sense, 5′-GGACTTGCTCACCAACCAAT-3′; CBG antisense, 5′-AGGCTTCCATCCCTCAAAGT-3′, the 302 bp amplicon; actin sense 5′-TGTCACCAACTGGGACGATA-3′ and actin antisense 5′-AGGGCAACATAGCACAGCTT, the 439 bp amplicon were used as internal PCR controls. PCR products were separated in agarose gels and stained with ethidium bromide (Amersham).

3. Results

CBG immunostaining was observed in a fraction of the neurons in the supraoptic nuclei (SON, Fig. 1a) and in the paraventricular nuclei (PVN, Fig. 1b) of the hypothalamus, including the retrochiasmatic portion of the SON (Fig. 1d). CBG-immunoreactive cells were almost exclusively magnocellular with a mean perikaryal diameter of approximately 15 μm. Immunoreactivity was in general relatively weak. Staining was often restricted to a granular reaction product in the perinuclear cytoplasm while the nuclei were devoid of specific staining. Numerous immunostained neuronal processes with varicosities appeared in the magnocellular hypothalamic nuclei (Fig. 1a and b) and in the lateral hypothalamus (Fig. 2b). The bed nucleus of the stria terminalis (BNST, Fig. 2d) and the preoptic region contained small groups of magnocellular CBG positive neurons. Scattered parvocellular neurons in the PVN, and the suprachiasmatic nucleus (SCN, Fig. 1c) showed weak immunostaining for CBG. Single magnocellular neurons in the periventricular nucleus of the hypothalamus contained clear cytoplasmic CBG immunoreactivity (Fig. 2a). Specific CBG staining appeared in single ependymal cells (Fig. 2f). A portion of the epithelial cells in the choroid plexus was also CBG positive. Immunocytochemical control sections remained unstained.

In the cerebellar cortex, we observed a portion of the Purkinje cells that stained with anti-CBG (Fig. 2h). In the granular layer of the cerebellum, there were numerous CBG-positive fibers, which appeared to be Mossy fibers (Fig. 2g). In the CA 2 (Fig. 2e) and CA 3 regions of the hippocampus we found numerous cells immunostained for CBG (Fig. 2e) which may include pyramidal and non-pyramidal cells. In the motor cortex we failed to find CBG-stained neuronal perikarya. Immunostained fibers occurred in the mesencephalon in the periaqueductal gray and in the medulla oblongata. The mesencephalon and the medulla oblongata, contained scattered

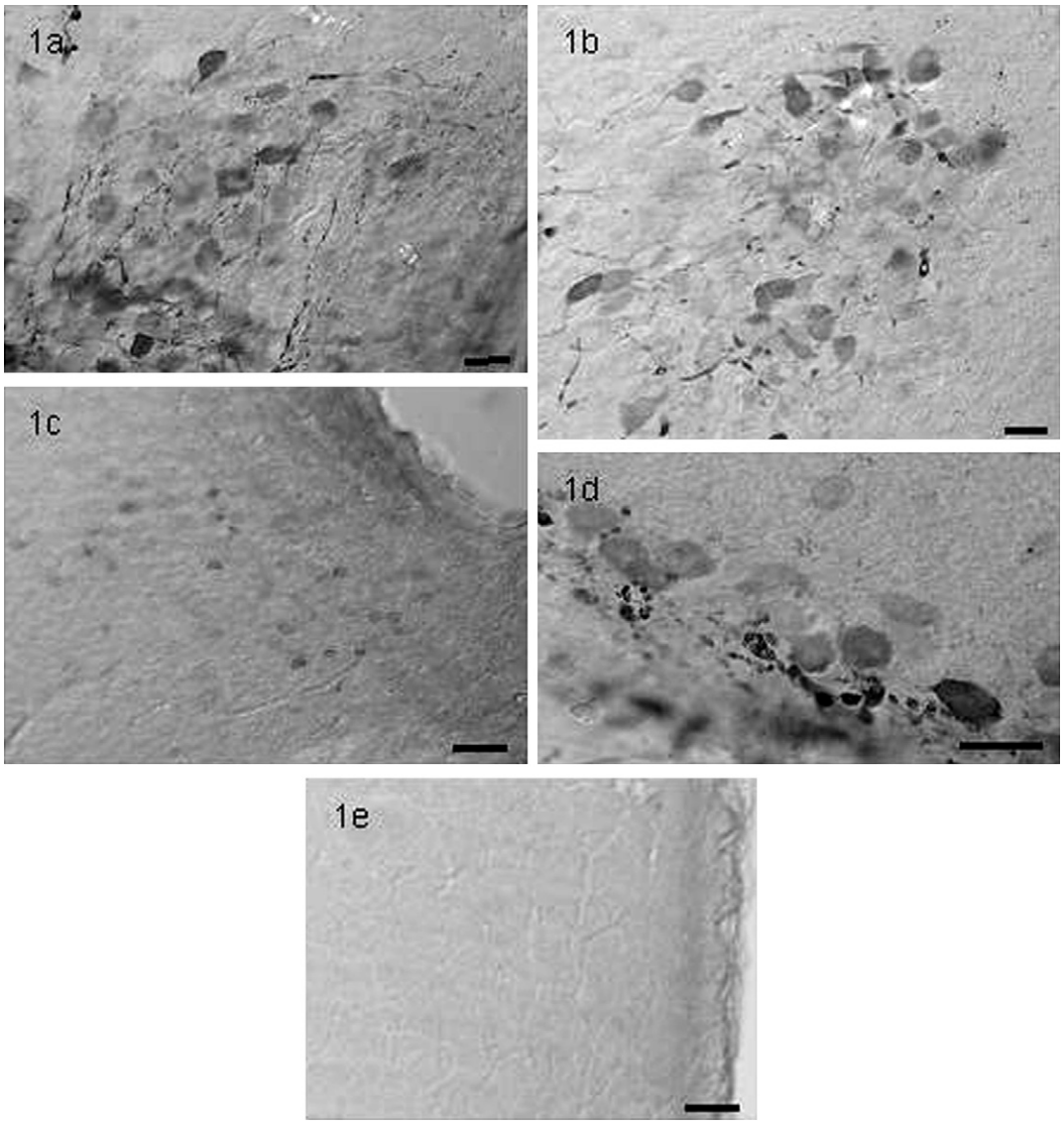

Fig. 1. CBG immunoreactive magnocellular perikarya and processes in the SON (a) and the PVN (b). Parvocellular CBG neurons appear in the SCN (c). The retrochiasmatic portion of the SON contains CBG positive perikarya and intensely stained axonal varicosities (d). Immunocytochemical control remained unstained (PVN, e). Scale bar = 20 μm.

CBG positive fibers in the periaqueductal grey and in the formation reticularis while CBG immunostained perikarya could not be observed in the brain stem. In the spinal cord we found single CBG-positive perikarya in the dorsal horn.

Immunostaining of sections of the posterior hypothalamus revealed CBG reactivity in axonal varicosities in both the internal and the external layer of the median eminence (ME, Fig. 2c). In the internal layer of the ME, we observed CBG-positive axon terminals in close apposition to blood vessels.

Semithin sections of the posterior pituitary lobe exhibited scattered CBG-positive Herring bodies while pituicytes were in all cases CBG negative. A portion of the endocrine cells in the intermediate and in the anterior lobe were CBG positive.

RT PCR of RNA extracts revealed clearly visible bands of the amplification product in the ethidium bromide gels. PCR product had an approximate size of 300 bp. In extracts of the paraventricular and supraoptic nuclei, the choroid plexus and the in median eminence, we observed clearly visible bands. Outside the hypothalamus, we obtained the most pronounced PCR signal in the hippocampus. The cerebellum contained clearly detectable amplification product while the cerebral cortex seemed to be free of CBG-encoding transcripts. The anterior pituitary lobe and the intermediate lobe showed clearly visible amplification product of CBG mRNA, no signal was found in the posterior lobe. The negative control, performed without template showed no signal while liver as a positive control contained strong signal. RT PCR of β-actin showed specific amplification product at 439 bp in all samples except for the negative control (Fig. 3).

4. Discussion

We recently found CBG in magnocellular neurons within the PVN, the SON and associated nuclei, partially co-localized

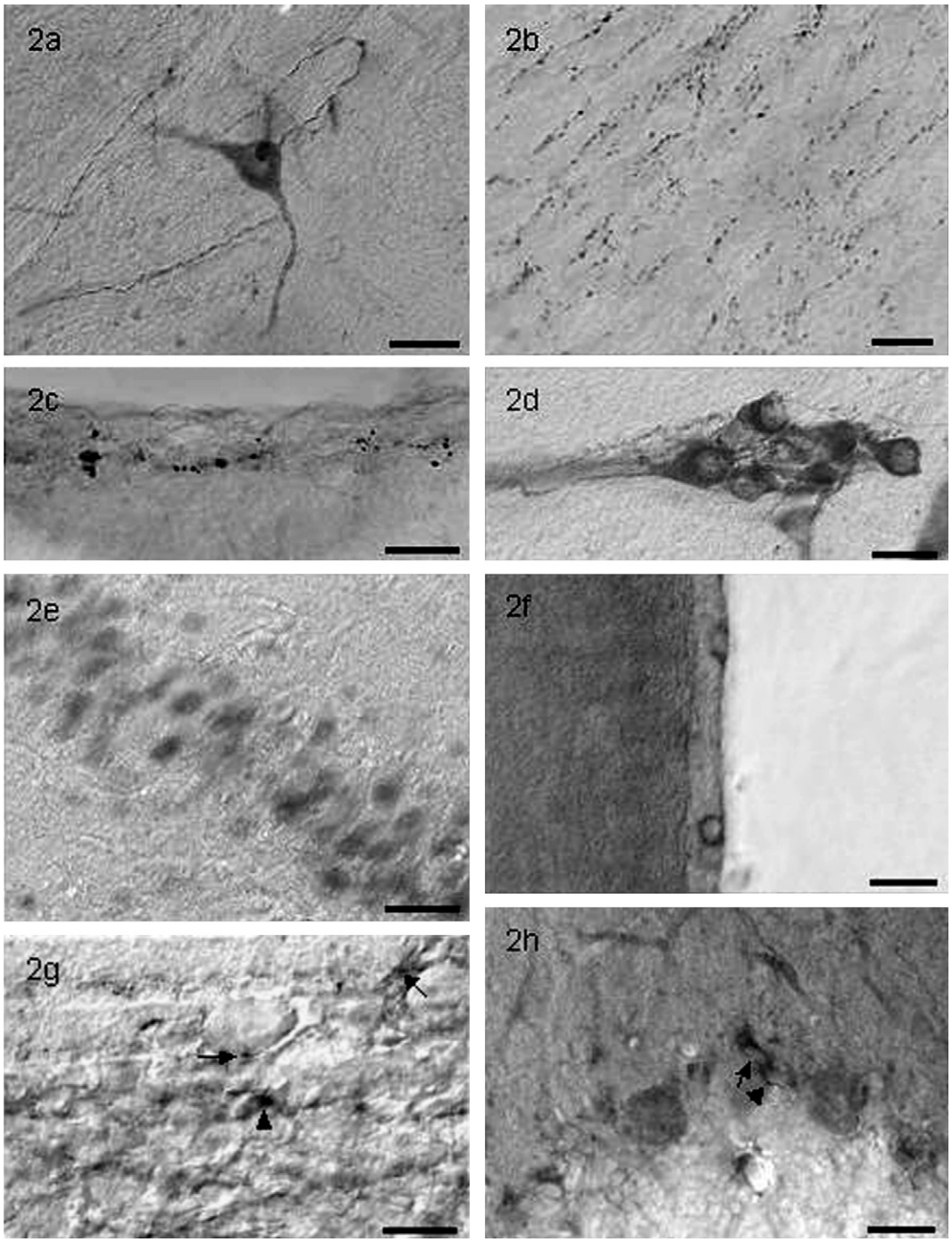

Fig. 2. Single magnocellular CBG neuron in the PEV (a). Numerous CBG immunostained neuronal processes with varicosities appear in the lateral hypothalamus (b). Single CBG stained projections can be observed in the internal layer of the median eminence (c). Small group of CBG immunoreactive neurons in the BNST (d). CBG immunoreactivity was observed in a portion of the pyramidal cells of the CA 2 region of the hippocampus (e). A small fraction of the ependymal cells was CBG positive (f). In the granular layer of the cerebellar cortex occur CBG positive nerve terminals (g). A portion of the Purkinje cells (h) and immunostained nerve fibers (arrows) can also be observed. Scale bar = 20 μm.

with the posterior lobe hormones VP and OT (Möpert et al., 2006). Numerous CBG-immunostained neuronal processes appeared in the lateral hypothalamus, the suprachiasmatic nucleus, the bed nucleus of the stria terminalis, the preoptic region and the arcuate nucleus. The localization of CBG in axons and axonal varicosities in the lateral hypothalamus, the median eminence and the posterior pituitary indicates a neurohormone-like transport and storage. The co-localization of the steroid-binding protein and the classical neuropeptides OT and AVP in neuroendocrine projections has been shown before (Möpert et al., 2006). Oxytocinergic and vasopressinergic neurons are known to project to the mesencephalon, the brain stem and the preoptic region (Sofroniew, 1983; Jirikowski et al., 1988). Since CBG is present in some of these neurons, the respective

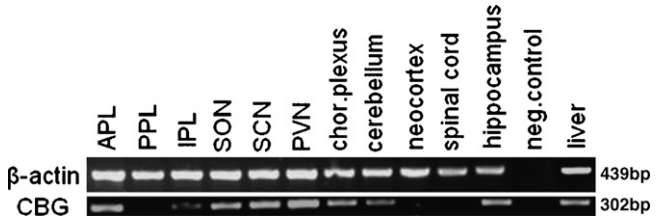

Fig. 3. Ethidium bromide gel of RT PCR for CBG emRNA in different rat brain regions: Specific amplification product for CBG at 300 bp is visible in the APL, anterior pituitary lobe; SON, supraoptic nucleus, SCN, suprachiasmatic nucleus; PVN, paraventricular nucleus; choroid plexus; cerebellum; hippocampus. RNA extracts from liver served as positive control. As a negative control PCR was run without template. The PPL, posterior pituitary lobe; IPL, intermediate pituitary lobe; neocortex and spinal cord did not contain CBG enconding mRNA. As a further control β-actin mRNA was examined in the same samples which shows specific bands at 439 bp.

projections are likely to exist for CBG as well. Our demonstration that colchicine treatment greatly increased CBG immunoreactivity in perikarya further supports the idea that under normal conditions CBG is subject to high secretory turnover. Whether CBG can be released from nerve terminals has not been shown so far. However, we demonstrated that sex hormone binding globulin (SHBG) is found in dense cored secretory vesicles by immuno electron microscopy (Herbert et al., 2006), which suggests that SHBG is released from terminal sites in brain and pituitary. A similar situation may be true for CBG.

The current study and Möpert et al. (2006) are the first demonstrations that CBG is expressed in rat central neurons, similar to SHBG. We have seen that SHBG expression is controlled by systemic estradiol levels (Gao et al., 2003). Whether CBG expression in brain is controlled by adrenal steroids is the topic of ongoing studies. In a recent study, we could demonstrate that CBG is also expressed in the human hypothalamus: Magnocellular and parvocellular hypothalamic neurons expressed CBG, together with oxytocin and vasopressin (Sivukhina et al., 2006). Whether extrahypothalamic areas are capable of CBG expression in humans is the topic of ongoing studies.

While CBG in the periphery may be important for control of the hypothalamo-piuitary-adrenal (HPA) axis (De Kloet et al., 1984), its role in brain has not been examined. However, it is reasonable to assert that CBG in brain is also involved in stress responses. Vasopressin is not only involved in control of ACTH release (Ma et al., 1997) but has also been associated with central stress response. Engelmann et al. (2000) demonstrated that the forced swim test released vasopressin from numerous brain areas while ‘‘social defeat’’ stress released oxytocin, suggesting that both vasopressin and oxytocin are involved in responses to stress, but that they may respond differently under different conditions. A primary function of OT is its central and systemic release during parturition (Seif and Robinson, 1978; Soloff et al., 1979; Fuchs et al., 1982), which is also a very stressful event. That CBG is co-localized with oxytocin and vasopressin, further suggests its role in central control of behavioural responses to stress.

The parvocellular PVN is the primary CRF-producing area of the brain (Swanson and Simmons, 1989; Makino et al., 1995) and therefore important for control of adrenocorticotropin release. Glucocorticoids are known to exert feedback control back on the brain via glucocorticoid receptors, GCR (De Kloet, 1984, 1995; Makino et al., 1995), but only few of the perikarya in the PVN actually contain GCR immunostaining (Jirikowski et al., 1993; Agnati et al., 1985). Swanson and Simmons (1989) found that corticosterone infusions into the brain activated CRF expression in the dorsal PVN indicating a responsiveness of these neurons to changing brain glucocorticoid levels. This may be mediated either through surrounding GCR positive interneurons or through indirect or non-genomic effects of the adrenal steroids.

We found CBG immunoreactivity in a distinct portion of ependymal cells lining the third ventricle. Breuner and Orchinik (2002) demonstrated CBG immunoreactivity in cells lining the cerebral ventricles, which they attributed to uptake of CBG from the CSF. Our in situ hybridization experiments failed to find CBG encoding mRNA in ependymal cells (Möpert et al., 2006), which would corroborate this assertion. The CSF has been shown to contain significant amounts of CBG in humans (Predine et al., 1984; Schwarz and Pohl, 1992). The functional importance of CSF CBG is unknown to date.

CBG immunoreactivity was evidenced in the internal layer of the median eminence (ME) in close apposition of blood vessels from where it may gain access to the portal circulation of the anterior pituitary lobe, along with classical releasing factors. Immuno electron microscopy for CBG in the ME is yet to be performed.

CBG immunostaining was observed in numerous endocrine cells of the anterior pituitary lobe, confirming our previous observations with in situ hybridization. An intrinsic expression of CBG in a portion of these cells is likely to occur (Möpert et al., 2006). Although we did not perform colocalization experiments with the anterior lobe peptides, the high number of stained cells, their morphology and distribution indicate that CBG immunoreactivity is not confined to corticotrophs as previously suggested (De Kloet et al., 1984) but may also occur in other endocrine cells.

CBG immunoreactivity was found in a portion of the Herring bodies in the posterior pituitary lobe (Möpert et al., 2006). This allows for the assumption that CBG, at least here, is processed for release. This agrees with our previous observation that SHBG is localized in secretory vesicles in Herring bodies along with OT (Herbert et al., 2003). The colocalization of CBG with either OT or VP in Herring bodies was shown in our recent study.

We have seen that central infusions of SHBG alter female sexual behaviors (Caldwell et al., 2000, 2002) in a manner that suggests that SHBG has a role in mediating steroid activation of the brain. We have suggested that there are membrane-associated receptors for SHBG in the brain (Caldwell, 2001, 2002). Strel’chyonok and Avvakumov (1991) have described a binding site for CBG on the cell membrane. Nakhla et al. (1988) have taken this idea further to suggest that CBG stimulation of its membrane-associated receptor activates intracellular adenylate-cyclase/cAMP second messenger systems. Both of these studies were done with peripheral tissues or cells. It is unknown

so far if there is a CBG membrane receptor in brain. It is possible that membrane-associated receptors also exist for CBG to mediate central effects on behaviors including stress response.

We discovered CBG immunoreactivity in the cerebellum. Nuclear GCR have been found in this brain region (Beaumont and Fanestil, 1983) suggesting that there may be glucocorticoid targets. CBG immunostaining occured in a portion of the Purkinje cells an in fibers in nerve terminals in the granular layer which are likely to be mossy fibers. CBG may have transmitter function in the cerebellar cortex, perhaps interacting with CRF (Swinny et al., 2004).

CBG in the spinal cord was found in single cells of the dorsal horn suggesting that CBG may play a role in modifying sensory perception. We failed to find CBG mRNA in the spinal cord with RT PCR. This may be due to low expression levels of CBG or the small numbers of CBG positive neurons in the spinal cord. Given the important role of neuraxially applied corticosteroids to treat spinal pain and other types of painful conditions (Gordon, 1980; Manchikanti, 2002) it may be that CBG plays a role in pain perception at the level of the spinal cord.

Clearly the different locations of CBG in the rat central nervous system indicate various different functions. In addition to being a factor in the hypothalamo- neurohypophysial system, CBG may be a central neuroactive substance and an anterior pituitary lobe hormone. Most likely the properties of central CBG exceed the function of a mere transporter for serum glucocorticoids.

Acknowledgement

Supported by NIH grant R21 MH 069810-01A1 to J.D.C. and G.F.J.

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Lewis JG, Möpert B, Shand BI, Doogue MP, Soule SG, Frampton CM, Elder PA. 2006.

Plasma Variation of Corticosteroid-binding Globulin and Sex Hormone-binding Globulin.

Horm Metab Res, 38(4): 241-245

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Plasma Variation of Corticosteroid-binding Globulin and Sex Hormone-binding Globulin

Abstract

Sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) circulate in plasma and bind their cognate ligands with high affinity, offering a steroid delivery system to target tissues by a variety of mechanisms. Analysis of these steroid-binding proteins is gaining importance in the clinical setting, although more information is warranted on their diurnal and biological variation. This study shows that plasma SHBG (in normal subjects) exhibits little diurnal or biological variation over the 30 day period studied, in contrast to CBG, where plasma levels peak in the early afternoon. This leads to attenuation of the diurnal free cortisol level rhythm compared to total cortisol. We also show that plasma CBG is significantly lower in male subjects with the metabolic syndrome compared to age-matched lean counterparts, and may therefore act as a surrogate marker of insulin resistance. The consequence of lower levels of CBG in these obese male subjects is reflected by higher levels of circulating free cortisol, potentially offering a more favourable environment for adipogenesis.

Key words
Cortisol · obesity · metabolic syndrome · CBG · SHBG

Introduction

Recent advances in knowledge of sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) beyond their steroid-binding properties are attracting interest. These roles include the relationships between these proteins and obesity, insulin resistance and plasma lipids. Plasma SHBG has been shown to correlate inversely with anthropometric measurements and insulin resistance in both females and males [1–5], while insulin inhibits the production of SHBG by Hep G2 cells *in vitro* [6]. Reduced plasma SHBG levels are also associated with low high-density lipoprotein (HDL) cholesterol levels in females [7]. In males, SHBG may affect the metabolism of HDL cholesterol [8], and is the central candidate protein in the hormonal regulation of the lipid profile, thereby influencing coronary heart disease risk [9].

Like SHBG, CBG secretion is reportedly inhibited by insulin [10]. It circulates at levels inversely proportional to the degree of insulin resistance and affects the relationship between adiponectin and cortisol [11]. CBG may be involved in the relationship between cortisol-driven obesity in animals [12] and humans [13], although its role as a marker of insulin resistance in obese males is less certain [4]. However, knowledge of biological steroid-binding protein variations is desirable before they can be used as markers of insulin resistance in a clinical or outpatient setting. Although SHBG has no diurnal variation in women [14], intra-individual variation for SHBG in postmenopausal women with type 2 diabetes is reported to be 14.5%, indicating that a subsequent sample must rise or fall by more than that value to be considered biologically significant [15,16]. This contrasts with men, where there are no data on intra-individual variation although significant diurnal rhythms have been reported [17] with elevations

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Received 2 August 2005 · **Accepted after revision** 21 October 2005

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Horm Metab Res 2006; 38: 1–5 © Georg Thieme Verlag KG Stuttgart · New York ·
DOI 10.1055/s-2006-925338 · ISSN 0018-5043

above the mesor over daylight hours and an evening decline reaching nadir in the early hours of the morning. There is uncertainty about the diurnal variation of CBG [18], and there are no reports on its biovariation. We sought to address some of these issues by analysing plasma CBG and SHBG from normal subjects sampled over daylight hours. In order to examine their biovariation, we also analysed fasting plasma CBG and SHBG in male subjects with the metabolic syndrome and age-matched normal controls at 8 AM on four occasions 10 days apart.

Here, we will demonstrate that there is no detectable daytime variation in SHBG, but CBG levels are significantly higher between 12:30 and 1:30 PM than at the other time points tested. This serves to attenuate the early afternoon free cortisol peak compared to total cortisol. The levels of CBG were significantly lower in the male metabolic syndrome cohort compared to their lean counterparts. The consequence of lowered CBG in the metabolic group is reflected by higher free cortisol levels, potentially enhancing and promoting adipogenesis.

Materials and Methods

Written informed consent was obtained from all subjects; the studies were approved by the Canterbury Ethics Committee, Christchurch, New Zealand.

Diurnal study subjects

Normal subjects (10 males and 10 females, aged between 20 and 40 years) were recruited from hospital staff, and were apparently fit and healthy. Subjects had no oral, inhaled or parenteral glucocorticoid exposure within the preceding 12 months, were not acutely ill, and avoided exercise or alcohol on the study day. Blood samples were collected into EDTA tubes from an indwelling catheter inserted in an arm vein, the plasma separated and samples stored at – 20 °C for analysis. Sampling commenced at 8 AM and a minimum of 12 samples were collected every half hour until 10:30 AM and then every hour until 5:30 PM.

Biovariation study subjects

Volunteers without diabetes (5 males) were recruited from a clinical database of subjects with the metabolic syndrome classified according to the ATP III criteria [19]. The subjects were selected if they possessed three or more of these criteria, with at least one subject from each of the four decades of life from 20 to 60 years included in the study. Five age-matched males with one or less of the APT III criteria were recruited as controls for the study. Fasting blood samples were collected in EDTA tubes between 8 – 9 AM on days 1, 10, 20, and 30. The plasma was separated and stored at – 30 °C in subject batches for analysis; each sample was assayed in duplicate.

Methods

Plasma SHBG and CBG were measured by in-house enzyme-linked immunosorbent assay (ELISA) [20,21]. Plasma cortisol was measured by ELISA [22], and free cortisol was calculated from total cortisol and CBG using Coolens' equation [23]. These analytes are unaffected by freeze/thaw cycles of EDTA plasma. Analysis of data was carried out using the statistical package SPSS version 11.5 (SPSS Inc. Chicago, IL) and Sigmastat 3.1 (Systat Software Inc. Point Richmond, CA). Diurnal SHBG and CBG data were analysed by repeated measures of analysis of variance followed by paired *t*-tests. Biological variation was calculated from analytical, intra-individual, and inter-individual components of variation (CV_A, CV_I and CV_G respectively) by nested ANOVA [24]. The reference change value (RCV) for significant changes in serial results ($p < 0.05$) in an individual subject was calculated using the formula 2.77 $(CV_A^2 + CV_I^2)^{1/2}$. The concept of biological individuality is expressed as the index of individuality (IoI) and derived from the ratio of intra-individual and inter-individual variation. Low IoI values (< 0.6) indicate that the dispersion of values for any individual will span only a small part of the reference interval where conventional population-based reference ranges would be inadequate for detecting whether a significant change has occurred [25]. Conversely, when the IoI is 1.4 or greater, the variation in an individual will fit population reference limits more closely, and would be sufficient for a screening test. Insulin resistance was determined using the homeostasis model assessment (HOMA) computer model from fasting plasma glucose and insulin measurements with results expressed as percentage sensitivity [26]. Low values indicate insulin resistance. Levels were compared between metabolic syndrome and lean males using paired *t*-tests or Wilcoxon's signed rank test as appropriate.

Results

Daytime plasma CBG variation is shown in Fig. **1 a** along with the corresponding total cortisol (Fig. **1 b**) and calculated free cortisol levels (Fig. **1 c**). There was a significant increase in circulating CBG at 12:30 and 1 PM compared to the mid-morning (8:30 – 10:00 AM) and late-afternoon (4:30 – 5:30 PM) time points ($p < 0.05$). This increase in CBG served to attenuate the early afternoon rise in plasma free cortisol compared to total cortisol.

Sex hormone-binding globulin data were analysed according to gender since males have significantly lower circulating SHBG levels than females, in contrast to CBG [20]. Plasma SHBG remained constant over the time course studied for both males and females (Fig. **2 a, b**, respectively). Statistically, there were no significant differences between any time points analysed according to gender or as a total group. CBG and SHBG both showed a Gaussian distribution in both males and females.

The biovariation results for CBG, total cortisol, calculated free cortisol and SHBG for male subjects with the metabolic syndrome and their controls are shown in Table **1**.

Discussion

Daytime CBG variation may have biological implications for daytime cortisol variation, with early afternoon rises in CBG attenuating the early afternoon surge of free cortisol relative to total plasma cortisol. This is most likely a subtle effect, but serves to illustrate the relevance of the dynamic assessment of free cortisol levels. Conversely, stress is known to reduce circulating CBG levels [27,28], so free cortisol would be amplified relative to total plasma cortisol. In a clinical setting, the physician may be more interested in free cortisol derived from the measurement of total

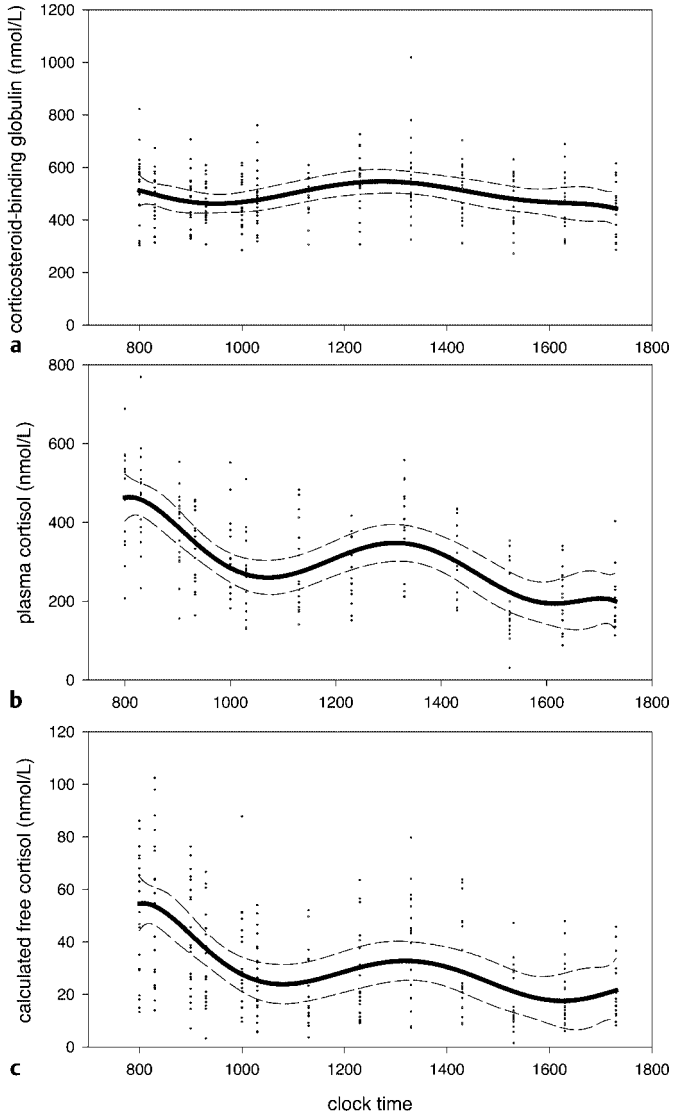

Fig. **1** Daytime variation in plasma corticosteroid-binding globulin (**a**), total plasma cortisol (**b**) and calculated free cortisol (**c**) in 20 normal subjects. Sixth order regression lines ± 95% confidence intervals are shown. Values are nmol/l.

cortisol and CBG. This is especially significant in cases where the diagnosis of adrenal sufficiency is paramount in guiding hydrocortisone replacement. This may occur in critical illness or septic shock, where low CBG levels are likely to confound the interpretation of adrenal sufficiency when based on total plasma cortisol levels [29, 30].

The absence of any detectable diurnal variation of SHBG over the time course studied for both males and females would not be expected to impact significantly on the normal diurnal variation of testosterone, whether total testosterone or the free fraction. Our results appear to be at variance with a report by Diver et al. [17], in which SHBG was shown to be at a nadir between 2 and 6 AM. However, our sampling schedule from 8 AM to 5 : 30 PM would not be expected to detect this trough. The rationale for using this sampling time frame was that it spanned the times when blood samples are usually taken in a clinical or outpatient setting.

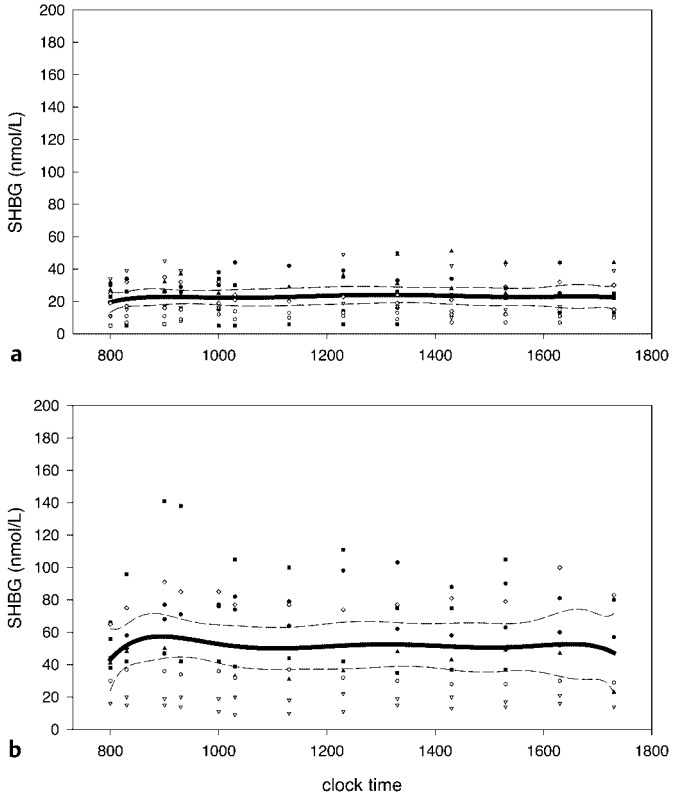

Fig. **2** Daytime variation of plasma sex hormone-binding globulin in 10 males (**a**) and 10 females (**b**). Sixth-order regression lines ± 95% confidence intervals are shown. Values are nmol/l.

Table **1** Plasma CBG, cortisol, calculated free cortisol and SHBG in metabolic syndrome and lean male subjects. Shown is the mean ± SD, reference change values (RCV), the index of individuality (IoI), homeostasis model assessment (HOMA) (% sensitivity) and body mass index (BMI). Significant comparisons ($p < 0.05$) are indicated using the paired *t*-test or Wilcoxon's signed rank test for non-normal distribution*

	Metabolic syndrome (n = 5)	***Lean (n = 5)***
CBG (nmol/l)	370 ± 65	561 ± 105 (p = 0.008)
CBG RCV (nmol/l)	187	674
CBG IoI	< 0.1	> 10.0
Cortisol (nmol/l)	450 ± 88	438 ± 158
Cortisol RCV (nmol/l)	212	159
Cortisol IoI	0.89	0.21
Free cortisol (nmol/l)	75 ± 29	54 ± 33
Free cortisol RCV (nmol/l)	56	68
Free cortisol IoI	0.65	0.75
SHBG (nmol/l)	25.6 ± 8.1	29.1 ± 9.4
SHBG RCV (nmol/l)	6.2	8.9
SHBG IoI	0.15	0.31
HOMA (% sensitivity)	57.2 ± 18.7	186.5 ± 144.8 (p = 0.008*)
BMI (kg/m^2)	30.7 ± 2.0	23.1 ± 3.6 (p = 0.02)

In the biovariation study, it is of interest that CBG was significantly lower in the metabolic syndrome males compared to their age-matched lean counterparts. We have previously reported an inverse association between SHBG and anthropometric markers in obese males [4,9], although not for CBG in the same obese cohort. In the current study, plasma levels of these steroid-binding proteins were compared between lean and metabolic syndrome male subjects with low and high body mass indexes, respectively, theoretically allowing a more favourable stratification. Although the intention of the study was to examine only the biovariation of the steroid-binding proteins, it is somewhat surprising to find significant differences in CBG levels between the male groups with only five subjects in each group. Although there were differences in the mean SHBG levels between the male groups, they failed to reach significance due to the low power of the study. It is of interest that the mean SHBG levels for the male metabolic and lean groups were similar to our previous findings [4,20], implying that the power to statistically discern these differences would increase with increased numbers in each group.

Although the biovariation study initially included a total of ten females, the results are not reported here since differences between the normal and obese groups were confounded by variable increases in SHBG and CBG due to oral contraceptive (OC) use [31,32] and the possibility of cyclic variation in some women [33,34]. A large cohort of females would be required to address these issues.

An interesting finding is that the RCV for CBG in males was higher in lean compared to obese males, suggesting that other factor(s) may govern circulating CBG levels. It is possible that sensitisation to these factor(s) is an important extra element in metabolic control in lean individuals. which could be partially lost in the metabolic syndrome. Furthermore, this control mechanism may be responsible for the similar RCVs in free cortisol observed in the metabolic and lean groups. Interestingly, the RCVs for SHBG were similar in the male groups.

In males, while there was no difference in total plasma cortisol levels between the metabolic and lean groups, the consequence of significantly lower CBG levels was reflected by higher levels of calculated free cortisol in the metabolic syndrome group. This is an interesting observation, supporting the concept of a role for free cortisol as a determinant of adipose tissue deposition [13,35]. Furthermore, enhanced 11β-hydroxysteroid dehydrogenase type 1 activity in central obesity may further amplify local glucocorticoid activity by pre-receptor metabolism of cortisone to cortisol in subcutaneous adipose tissue [36].

The use of the Coolens' equation to calculate free cortisol levels may tend to overestimate free cortisol since the free cortisol to total cortisol fraction appears to be slightly higher than expected by direct measurement, usually 6% or less of the total plasma cortisol [37]. Notwithstanding this limitation, the conclusions remain unaltered since comparison within and across sample groups would be expected to be similarly affected.

The index of individuality (IoI) for total cortisol, calculated free cortisol and SHBG across the male groups was low, demonstrating that conventional population-based reference intervals are of limited value in the detection of unusual results for a particular individual. However, the IoI for CBG in the lean males was much higher compared to metabolic males, offering support to the concept of a sensitive control mechanism for plasma CBG levels in lean males.

An important finding of this study is that while SHBG exhibits little diurnal variation, CBG rises in the early afternoon resulting in attenuation of the daytime rhythm of free cortisol. Another significant finding in the biovariation arm of the study is that males with the metabolic syndrome have significantly lower plasma CBG levels compared to their lean counterparts. This results in higher circulating free cortisol levels that may predispose to adipogenesis.

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5 Diskussion

5.1 Das CBG im zentralen Nervensystem

Diese Arbeit zeigt erstmalig spezifische Immunoreaktivität von CBG auf zellulärer Ebene im zentralen Nervensystem der Ratte. Die Ergebnisse der In Situ-Hybridisierung auf Semidünnschnitten und der RT-PCR von homogenisiertem Hypothalamusmaterial sprechen für eine intrinsische Expression des Proteins. Auf Grund seiner Größe ist es unwahrscheinlich, dass CBG die Blut-Hirn Schranke überwindet um in den Zellen zu akkumulieren (Pardridge et al. 1986). Die Lokalisation von CBG in Axonen und axonalen Varikositäten im lateralen Hypothalamus, der Emenentia mediana sowie im Hypophysenhinterlappen weisen darauf hin, dass CBG wie ein Neurohormon transportiert und möglicherweise gespeichert und ausgeschüttet wird.

Frühere Experimente konnten die Existenz von CBG im Hypothalamus nicht bestätigen (Kuhn et al. 1986). Bei diesen Studien wurden die Tiere jedoch nicht mit Colchizin vorbehandelt. Colchizin stört die Bildung von Mikrotubuli und damit den axonalen Transport in der Zelle. Nach der intraventrikulären Behandlung der Tiere mit Colchizin zeigte sich eine deutliche Akkumulation von CBG in den entsprechenden Zellen. Dies stützt die Annahme, dass CBG einem hohen sekretorischen Turnover unterliegt und axonal transportiert wird.

Ob CBG selbst an Nervenendigungen ausgeschüttet wird konnte bisher nicht bewiesen werden. Elektronenmikroskopische Experimente demonstrierten die Koexistenz des funktionsverwandten Steroidbindungsproteins SHBG mit OT in sekretorischen Vesikeln der Axonvarikositäten des Hypophysenhinterlappens (Herbert et al. 2006). Dies erlaubt die Vermutung, dass SHBG ähnlich wie ein Peptidhormon prozessiert und ausgeschüttet wird. Da wir mittels Immunhistochemie zeigen konnten, dass CBG in den Projektionen der Neurohypophyse und in einem Teil der dort befindlichen Herringkörper vorhanden ist, könnte für CBG ein ähnlicher Mechanismus bestehen. Sollte CBG wie SHBG in sekretorischen Vesikeln vorkommen, was Gegenstand momentaner Untersuchungen ist, würde es die Hypothese über die Funktion des CBG als Neuromodulator oder gar als Neurotransmitter stützen. Dabei könnte es sehr wohl auch die Rolle eines hypophyseotropen Faktors einnehmen und damit die Ausschüttung der in der Hypophyse (HP) produzierten Hormone beeinflussen.

CBG fand sich in magnozellulären Neuronen des Hypothalamus insbesondere im NPV, dem NSO und deren assoziierten Kerngebieten. Mit kombinierter Immunoperoxidasefärbung und Immunofluoreszenz beobachteten wir eine Koexistenz von CBG und OT, und in geringerem Maße von CBG und VP, in magnozellulären Neuronen des Hypothalamus. Dabei zeigte sich, dass etwa 30% der oxytocinergen Zellen auch positiv für CBG waren. Oxytocinerge und vasopressinerge Neurone projizieren mit axonalen Fortsätzen in das Mittel- und Stammhirn sowie in die präoptische Region (Sofroniew 1980). Da CBG in einigen dieser Neurone vorhanden ist und wir zudem zahlreiche CBG-immunopositive Axone im Stamm-, Mittel- und Zwischenhirn beobachten konnten, existieren möglicherweise äquivalente Projektionen auch für CBG.

Seit einiger Zeit verdichten sich die Hinweise darauf, dass die Funktion des CBGs weit über die eines einfachen Steroidtransporters hinausgeht. Welche Rolle das CBG dabei im Gehirn einnehmen könnte, ist weitgehend ungeklärt. Es ist wahrscheinlich, dass die Lokalisation, die Verteilung und die Expression von CBG in oxytocinergen und vasopressinergen Neuronen von funktioneller Bedeutung ist.

Die beiden Neuropeptide OT und VP sind an den zentralen Mechanismen der Stressantwort beteiligt und regulieren stressbedingtes Verhalten, wobei sie unter verschiedenen Bedingungen unterschiedlich reagieren. Über membranassozierte Rezeptoren vom V1b-Subtyp verstärkt VP die CRH-Wirkung an corticotropen Zellen der Adenohypophyse und beeinflusst so die Ausschüttung von ACTH (Aguilera und Rabadan-Diehl 2000; Volpi et al. 2004). OT hingegen scheint eine zentral gesteuerte angst- und stresslösende Wirkung zu besitzen (Neumann et al. 2000, Panksepp et al. 1997; Windle et al. 1997). Stress selbst wiederum beeinflusst sowohl die Expression als auch die Ausschüttung von OT und VP im Gehirn (Jezova et al. 1995, Lang et al. 1983). Neuere Untersuchungen weisen darauf hin, dass Glucocorticoide als Teil der endokrinen Stressantwort diese Regulation vermitteln (Lauand et al. 2007, Ruginsk et al. 2007). Es ist denkbar, dass CBG Einfluss auf die stressmodulierenden Eigenschaften von OT und VP hat. Es könnte die Wirkung der Steroide auf die oxytocinergen und vasopressinergen Neurone regulieren, indem es die Hormone an die Zellen transportiert und als Puffer, sowohl intra- als auch extrazellulär, deren Verfügbarkeit steuert. Dies setzt das Vorhandensein klassischer GR voraus. Da diese jedoch nur in einem Teil der oxytocinergen Zellen des HT und des limbischen Systems

vorhanden sind (Jirikowski et al. 1993), könnte CBG auch selbst als neuroaktive Substanz fungieren und die Ausschüttung der beiden Peptide beeinflussen.

Eine mögliche Funktion des CBG im Allgemeinen ist also, die Bioverfügbarkeit von freiem und damit, entsprechend der „free hormone hypothesis“, biologisch aktivem CORT zu steuern (Breuner und Orchinik 2002). Es könnte als extra- und intrazelluläres Protein die potentiell zytotoxische Wirkung von hohen Glucocorticoidkonzentrationen auf Neurone (Sapolsky 1993) abpuffern, als Vorratsspeicher für schnell verfügbares CORT fungieren und die Fraktion des freien CORT innerhalb der Zelle steuern. Auf diesem Weg hätte es unter anderem einen indirekten Einfluss auf Feedbackmechanismen der Steroide im Hypothalamus.

Der NPV ist der Hauptproduktionsort des CRH und damit die oberste Kontrollinstanz für die ACTH-Ausschüttung in der Hypophyse (Makino et al. 1995). Es ist bekannt, dass Glucocorticoide dosisabhängig via nukleären Rezeptoren Feedbackmechanismen auf den Hypothalamus, und hier im Besonderen auf die CRH-Zellen, ausüben (Kretz et al. 1999, Swanson und Simmons 1989). Einige der Neuronen im NPV besitzen nukleäre GR (Agnati et al. 1989) und es ist möglich, dass CBG, produziert in diesen oder in benachbarten Zellen, Glucocorticoide an die CRH-Zellen transportiert.

Swanson und Simmons (1989) fanden heraus, dass die Injektion von Cortiosteroiden in das Gehirn der untersuchten Ratten die CRH Expression im NPV dosisabhängig reguliert. Berdusco et al. (1995) zeigten an fetalen hypophysären Zellkulturen von Schafen, dass CBG die negative Feedbackwirkung von CORT auf die CRH-induzierte ACTH-Ausschüttung abschwächte. Betrachtet man diese Beobachtungen zusammen mit der morphologischen Verteilung des CBG im Hypothalamus ergibt sich ein weiterer möglicher Wirkmechanismus zerebralen CBGs. Es könnte die intrazelluläre Corticosteroidkonzentration abpuffern und damit die Bindung an intranukleäre Rezeptoren limitieren. Das würde auch teilweise die dosisabhängige Hemmung der CRH-produzierenden Zellen durch Glucocorticoide erklären.

Kuhn (1988) und Nakhla et al. (1988) zeigten, dass CBG aktiv von Zellen internalisiert werden kann. Es ist also über den reinen Transport der Steroide an die Zellen hinaus denkbar, dass CBG deren intrazelluläre Konzentration bei Bedarf erhöht; und dies sowohl schneller, als auch in stärkerem Maße, als ausschließlich durch Diffusion erreichbar wäre. Durch eine Veränderung der Anzahl der CBG-Transporter könnte dieser Mechanismus die zelluläre Glucocorticoidkonzentration auch auf einem zell-individuellen Level regulieren (Breuner und Orchinik 2002) und damit die Glucocorticoidwirkung im ZNS spezifischer machen.

Die Vorstellung über die Rolle des CBG im Organismus hat sich in den letzten Jahren zum Teil grundlegend geändert. Membranassoziierte Bindungsstellen für CBG mit Rezeptoreigenschaften wurden für periphere Gewebe postuliert (Hryb et al. 1986, Singer et al. 1988, Strel'chyonok und Avvakumov 1991, Maitra et al. 1993). Nakhla et al. (1988) zeigten, dass die Bindung von CORT-beladenem CBG an diesen Rezeptor das intrazelluläre Adenylatcyclasesystem aktiviert, die cAMP Konzentration innerhalb der Zelle steigert und somit eine Second-Messenger Kaskade initiiert. Dieser Mechanismus wird für die allgemein bekannten, schnellen, nichtgenomischen Steroidwirkungen an Zellen verantwortlich gemacht. Ähnliche Verhältnisse könnten auch im zentralen Nervensystem vorliegen. So könnte CBG zusammen mit CORT über einen CBG-Rezeptor in einer möglicherweise sehr viel schnelleren Weise Feedbackwirkung auf die CRH-Zellen ausüben, als es über einen Kernrezeptor denkbar wäre.

Für CBG und das funktionsverwandte Steroidbindungsprotein SHBG konnte gezeigt werden, dass ausschließlich die Bindung des Steroid-Proteinkomplexes das Second-Messenger System aktiviert. Die alleinige Bindung von CBG resp. SHBG blieb ohne Effekt (Nakhla et al. 1988, Hryb 1990). Dies lässt die Spekulation zu, dass CBG möglicherweise als Prohormon existiert und durch die Bindung von CORT aktiviert wird (Rosner et al. 1988, Nakhla et al. 1988) und stützt die weiter oben diskutierte Annahme, dass CBG als neuroaktive Substanz im zentralen Nervensystem fungiert.

CBG findet sich in der Innen- und in der Außenzone der Emenentia mediana. Über die ventrikelnahe Tanozytenschicht hat es damit Kontakt zum Hypothalamo-adenohypophysären Portalkreislauf. Auf die Bedeutung des CBG als möglichen hypophyseotropen Faktor wurde bereits eingegangen.

Zahlreiche Zellen des Hypophysenvorderlappens erschienen immunopositiv für CBG, was frühere Beobachtungen bestätigt (de Kloet et al. 1984, Kuhn et al. 1985). Wir konnten jedoch mittels RT-PCR auch eine intrinsische Expression des Proteins nachweisen. Wie schon erwähnt zeigten Berdusco et al. (1995), dass CBG die negative Feedbackwirkung von CORT auf die ACTH-Ausschüttung in der Hypophyse abschwächte. Zusätzlich ist mittels der oben beschrieben möglichen Modulation der VP-Ausschüttung, welche ihrerseits über einen V1b-Rezeptor die Wirkung von CRH auf die corticotropen Zellen verstärkt (Aguilera und Rabadan-Diehl 2000; Volpi et al. 2004), eine indirekte Beeinflussung der ACTH-

Ausschüttung denkbar. Dies unterstreicht, dass CBG auf mehreren Ebenen und mittels unterschiedlicher Mechanismen in die Vorgänge der endokrinen Stressantwort eingreifen könnte.

Obwohl wir keine Kolokalisationsexperimente mit den glandotropen Hormonen des Hypophysenvorderlappens durchgeführt haben, weisen die hohe Anzahl, die Morphologie und die Verteilung der angefärbten Zellen darauf hin, dass sich CBG nicht nur, wie vorher beschrieben (Perrot-Applanat 1984), in den corticotropen Zellen befindet, sondern auch in Zellen anderer Spezifität.

Wir fanden CBG positive Immunoreaktivität in einzelnen, das Ventrikelsystem auskleidenden, Ependymzellen. Die In Situ-Hybridisierung zeigte keine Reaktion in den entsprechenden Zellen. Das Protein wird hier also nicht exprimiert. Predine et al. (1984) konnten CBG im Liquor nachweisen. Es ist demnach möglich, dass die Ependymzellen das CBG an den Liquor abgeben oder es aus ihm aufnehmen. Es bleibt die Frage, welchen Ursprungs das CBG hier ist und durch welchen Mechanismus es in den Liquor gelangt. Dafür gibt es zwei Möglichkeiten: 1. Die Ependymzellen nehmen das CBG benachbarter periventrikulär gelegener Neurone auf und geben es in den Liquor ab oder 2. die periventrikulär gelegenen Neurone selbst schütten CBG in den Liquor aus. Die zweite Annahme würde sich mit unseren Beobachtungen decken, dass sich sehr dicht unter dem Ependym CBG-positive Neurone befinden. Gestützt wird diese Vorstellung dadurch, dass Liquorkontaktneurone existieren, welche nachweislich bioaktive Substanzen in den Liquor abgeben (Vigh et al. 2004). Ob das CBG unter diesen Stoffen ist, welche Funktion es im Liquor einnimmt und welche Bedeutung sein Vorkommen in Ependymzellen hat ist momentan noch unklar.

Die Western Blot Analyse von homogenisiertem Hypothalamus- und Hypophysenmaterial zeigte spezifische Banden bei einem Molekulargewicht von etwa 50 kDa. Das entspricht dem bekannten Molekulargewicht des Ratten-CBG von 46kDa. Die durchgeführte RT-PCR von Geweben verschiedener weiterer Gehirnregionen bestätigte diese Ergebnisse und wies nochmals auf eine intrinsische Expression von CBG in Neuronen hin. Auch ergab sich durch Kontrollexperimente mit Lebergewebe, dass das CBG, welches in der Leber produziert wird das gleiche Molekulargewicht besitzt, wie zentrales gebildetes CBG und auch durch die gleiche mRNA kodiert wird. Somit scheinen zentral und peripher produziertes CBG identisch zu sein.

5.2 Das CBG im Blutplasma

Aufgrund seiner morphologischen Verteilung und der neuen Erkenntnisse über seinen Charakter nimmt das CBG wahrscheinlich eine aktive Funktion innerhalb der komplexen Vorgänge der zentralen Stressantwort ein. Diese könnte, wie weiter oben dargelegt, auf verschieden Wegen und durch unterschiedliche Mechanismen vermittelt werden. Jedoch spielt auch das im Plasma zirkulierende CBG eine mitunter aktive Rolle in der Steuerung der Plastizität der Antwort eines Organismus auf Stress, indem es die Konzentration des freien, und damit biologisch aktiven, CORT reguliert. Dies wurde in Stressexperimenten mit Haussperlingen demonstriert (Breuner und Orchinik 2002, Breuner und Orchinik 2001). Es zeigte sich, dass der Plasmaspiegel von Gesamt-CORT und CBG saisonbedingt fluktuierte, während die Fraktion des freien CORT nahezu gleich blieb. Bei Ratten fand sich eine gewisse tageszeitliche Veränderung der CBG-Konzentration im Serum (Hsu und Kuhn 1988). Für den Menschen wurden derartige Untersuchungen bislang nicht durchgeführt. Dies war Aufgabe des zweiten Teils dieser Arbeit.

Wir beobachteten eine tageszeitliche Schwankung von CBG im Plasma des Menschen mit einem signifikanten Anstieg am frühen Nachmittag. Diese ging mit einer Abschwächung des nachmittäglichen Anstiegs der Konzentration des freien CORT, relativ zum Gesamt-CORT, einher. Das bedeutet, dass das CBG den Anteil des frei zirkulierenden Hormons abpuffert und gleichzeitig den potentiell verfügbaren Pool im Plasma erhöht. Bei den beschriebenen Versuchen mit Sperlingen wurde postuliert, dass diese während der Brutsaison auf ein größeres Reservoir von Glucocorticoiden angewiesen sind, da die Vögel einem gesteigerten Energiebedarf auf Grund eines höheren metabolischen Umsatzes gerecht werden müssen (Breuner und Orchinik 2002). Dieses Reservoir wird während der Zeit der Brutsaison durch einen erhöhten Spiegel von CBG und Gesamt-CORT garantiert ohne dabei die HPA-Achse aktivieren zu müssen. Ob das erhöhte CBG am frühen Nachmittag beim Menschen eine ähnliche Reservoirfunktion einnimmt ist ungeklärt.

Durch die Messung der CORT-Gesamtkonzentration und des CBG-Spiegels im Plasma ergibt sich eine Möglichkeit, die Konzentration von freiem (und damit biologisch aktivem) CORT im Plasma zu bestimmen. Die freie Fraktion des CORT korreliert negativ mit der CBG-Konzentration, was sowohl unsere Studie als auch Untersuchungen an kritisch kranken

Patienten demonstrierten (Savaridas 2004, le Roux 2003). Bei schwerer Krankheit und besonders im septischen Schock werden den Patienten Glucocorticoide von außen zugeführt, um einer potentiellen Nebenniereninsuffizienz (NNRI) entgegenzuwirken (Annane 2005). Die Diagnose der NNRI stützt sich dabei auf die Messung der CORT-Gesamtkonzentration im Plasma. Hamrahi et al. (2004) zeigten aber, dass trotz eines verminderten CORT-Gesamtspiegels bei schwer kranken Patienten die Fraktion des freien CORT im Vergleich zu einer Kontrollgruppe mehrfach erhöht war. Verantwortlich hierfür scheint ein signifikanter Abfall des Serum-CBG zu sein, wie er von Savaridas et al. (2004) bei schwer erkrankten Patienten beschrieben wurde. Es ist also festzuhalten, dass die Bestimmung der Fraktion des freien CORT, welche sich durch die Messung des Plasma CBG- und des CORT-Gesamtspiegels bestimmen lässt, besser geeignet wäre eine eventuelle Substitution mit Hydrocortison zu steuern, als die alleinige Bestimmung der CORT-Gesamtkonzentration.

6 Schlussfolgerungen

Diese Arbeit zeigt erstmalig, dass CBG in Zellen des zentralen Nervensystems der Ratte vorkommt und exprimiert wird.

Betrachtet man die gewonnenen Ergebnisse im Kontext der „free hormone hypothesis“ ist es möglich, dass CBG als intra- und extrazelluläres Puffersystem die Konzentration von freiem und somit biologisch aktivem CORT steuert. In der Konsequenz könnte es als Reservoir für schnell verfügbares und Puffer für potenziell überschüssiges CORT fungieren sowie die Wirkung von CORT an Zellen spezifischer gestalten. Hieraus resultierende Funktionen wären mannigfaltig.

CBG findet sich in Neuronen des NPV, dem Hauptproduktionsort des CRH, sowie in Zellen unterschiedlicher Morphologie innerhalb der HP. Denkbar ist, dass es hier in Feedbackmechanismen von CORT auf HT- resp. HP-Ebene eingreift.

OT und VP kommen gemeinsam mit CBG in Zellen des HT vor. Es ist möglich, dass CBG die Wirkung dieser beiden Peptide innerhalb der Stressantwort beeinflusst, indem es die Wirkung von CORT an OT- bzw. VP-Zellen steuert oder als neuroaktive Substanz deren Ausschüttung moduliert.

CBG findet sich in Projektionen und Axonvarikositäten in verschiedenen Regionen des ZNS. Die intraventrikuläre Behandlung der Tiere mit dem Spindelgift Colchizin steigerte die Färbeintensität der entsprechenden Zellen. CBG unterliegt demnach einem hohen sekretorischen Turnover und wird wie ein Neurohormon axonal transportiert. Da auch Projektionen innerhalb der Emenentia mediana CBG enthalten, könnte es die Rolle eines hypophyseotropen Faktors einnehmen.

Gemäß der Hypothese über die Existenz einer membranassoziierten rezeptorähnlichen Bindungsstruktur für CBG könnte CBG in Analogie zur Peripherie auch im zentralen Nervensystem über die Aktivierung von Second-Messenger-Kaskaden die schnellen nicht-genomischen Wirkungen von CORT an Zellen vermitteln. Möglich wäre eine schnelle Feedbackwirkung von CORT auf CRH-Zellen. Da ausschließlich CORT-beladenes CBG Second-Messenger-Kaskaden an den entsprechenden Rezeptorstellen initiiert ist es denkbar, dass CBG als Prohormon fungiert. Gestützt wird diese Annahme durch den neurohormonähnlichen Transport und die entsprechende Speicherung.

Vereinzelte Zellen des Ependyms enthalten CBG ohne es zu exprimieren. Ob diese Zellen das Protein von benachbarten Neuronen aufnehmen und an den Liquor abgeben oder es aus dem Liquor assimilieren ist bislang unklar.

Die Untersuchung der CBG-Konzentration im Plasma des Menschen ergab eine tageszeitliche Schwankung mit einem Maximum zwischen 12:30 Uhr und 13:00 Uhr. Diese Konzentrationserhöhung scheint verantwortlich zu sein für den verminderten Anstieg der freien CORT-Fraktion im Serum, verglichen mit der CORT-Gesamtkonzentration. Dieser Mechanismus erhöht den verfügbaren Pool an CORT und erfüllt möglicherweise eine Reservoirfunktion.

Durch die Messung der CORT-Gesamtkonzentration und des CBG-Spiegels im Plasma ergibt sich eine Möglichkeit, die Konzentration von freiem (und damit biologisch aktivem) CORT im Plasma zu ermitteln. Die Bestimmung der Fraktion des freien CORT ist möglicherweise besser geeignet eine eventuelle Substitution mit Hydrocortison zu steuern als die alleinige Bestimmung der CORT-Gesamtkonzentration.

Zusammenfassend lässt sich sagen, dass CBG der Rolle eines einfachen Steroidtransporters nicht gerecht wird. Unsere Beobachtungen verbunden mit neuesten Erkenntnissen anderer Forschungsgruppen zeigen, dass CBG das Potential einer neuroaktiven Substanz hat und seine Funktion in komplexen Vorgängen, wie der Stressantwort, ausübt.

7 Literaturverzeichnis

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8 Anhang

8.1 Lebenslauf

Name	Benjamin Möpert
Geburtsdatum, -ort	08.02.1980, Brandenburg/Havel
Familienstand	ledig

AUSBILDUNG

10/2000 - 10/2007	**Friedrich-Schiller-Universität Jena** Studium der Humanmedizin, Abschlussnote gut (2,16)
seit 10/2003	Doktorand am **Institut für Anatomie 2** Teichgraben 7, 07740 Jena Promotionsthema: *„Expression und funktionelle Implikationen des Corticosteroid-bindenden Globulins/CBG“*
09/1992 - 05/1999	**Friedrich-Schiller-Gymnasium**, Königs Wusterhausen Abschluss: Abitur, Note: gut (1,6)

AUSLANDSERFAHRUNG

06-09/07	3. Tertial des Praktischen Jahres in der Abteilung für Chirurgie im Spital Thun, Schweiz
06/2004 - 09/2004	**Steroid and Immunobiochemistry Unit der Canterbury Health Laboratories in Cristchurch, Neuseeland** Forschungsaufenthalt im Rahmen der Promotion als Boehringer Ingelheim Fellow
02-03/2003	Famulatur der Inneren Medizin am A.Ö. Krankenhaus Tamsweg, Österreich
09-10/2001	Famulatur am Austin Medical Center in Minnesota, USA
08/1996 - 06/1997	**Blooming Prairie High School** in Minnesota, USA

FACHBEZOGENE PRAKTIKA

02-06/07	2. Tertial des Praktischen Jahres in der Abteilung für Gastroenterologie des SHK Weimar
10/06 - 02/07	1.Tertial des Praktischen Jahres in der Abteilung für Anästhesiologie und Intensivmedizin am WKG Gera
02 - 03/2006	Famulatur der Anästhesiologie am Krankenhaus Gera
02 - 03/2004	Famulatur der Neurologie am Universitätsklinikum Jena
02 - 03/2001	Pflegepraktikum am Achenbach Kreiskrankenhaus Königs Wusterhausen

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PUBLIKATIONEN

Jirikowski GF, Pusch L, Möpert B, Herbert Z, Caldwell JD. Expression of corticosteroid binding globulin in the rat central nervous system. J Chem Neuroanat. **2007**. 34(1-2):22-28.

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STUDIENBEGLEITENDE TÄTIGKEITEN

10/2003 - 06/2004 **Friedrich-Schiller-Universität Jena**
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Tutor für mikroskopische Anatomie

Benjamin Möpert Februar 08

8.2 Danksagung

Ich möchte mich an dieser Stelle bei allen Personen und Organisationen bedanken, die mich bei meiner Arbeit unterstützt und damit die Entstehung dieser Promotionsschrift erst möglich gemacht haben.

An erster Stelle richtet sich mein Dank an Herrn Prof. Dr. Gustav F. Jirikowski, der mich ein ganzes Studienleben lang begleitete. Ich danke ihm für die freundliche Überlassung des Themas und seine immerwährende Geduld mit mir. Besonders dankbar bin ich darüber, dass er mich ermutigte und dabei unterstützte ein Stipendium anzustreben, welches es mir ermöglichte 3 wunderbare Monate in Christchurch, Neuseeland zu verbringen um dort an einem Projekt zu arbeiten.

Spezieller Dank gilt Zsófia Herbert; und dies auf vielen Ebenen. Diese Arbeit würde es ohne die Zusammenarbeit mit ihr nicht geben; ebenso wie meine Vorliebe für Bergkäse und portugiesischen Rotwein. Vielen Dank liebe Zsófi.

John Lewis und seinem gesamten Team vom Steroid Lab. in Christchurch, Neuseeland möchte ich danken für die herzliche Aufnahme in ihren Arbeitskreis, für die Überlassung eines blendend vorbereiteten Projektes und für seine Begeisterung an der Sache, die mich mehr als ein Mal ansteckte. An dieser Stelle möchte ich Boehringer Ingelheim für die finanzielle Unterstützung danken, die diesen Aufenthalt ermöglichte. Die Arbeit und die Menschen in Neuseeland haben mich begeistert und nachhaltig geprägt. Und wo sonst kann man schon mit Meerblick Snowboarden?!

Besonders dankbar bin ich auch Frau Roswitha Kasch, der guten Seele des Labors. Sie hält die Dinge zusammen, hat mir oft unter die Arme gegriffen und nicht selten eine zündende Idee gehabt.

Für ihre Zusammenarbeit und kreative Diskussionen danke ich Larissa Pusch, Prof. Erdogan Sendemir, Prof. Dr. Halbhuber und vielen anderen, die hier nicht namentlich erwähnt sind.

Für ihre moralische Unterstützung, die beim Abfassen einer solchen Arbeit wesentlich ist danke ich Florian Barthels, Sascha Eilert, Birte Möpert, Irina Baumbach und vielen mehr, die ich hier nicht alle nennen kann.

Sebastian Merz danke ich für das Korrekturlesen und die kurzfristige und sehr treffende konstruktive Kritik, Olena Kubyshkina für ihre Hilfe in der Endphase.

Zuletzt möchte ich mich herzlich bei meinen Eltern Martin und Hayk-Sten Möpert bedanken, die während dieser Arbeit und des gesamten Studiums vorbehaltlos hinter mir standen, mich immer wieder ermutigten und mich nicht nur finanziell unterstützten.

8.3 Ehrenwörtliche Erklärung

Hiermit erkläre ich, Benjamin Möpert, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Prof. Dr. G.F. Jirikowski, John G. Lewis, Zófia Herbert, Larissa Pusch, Roswitha Kasch, Prof. Erdogan Sendemir.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Bei der vorliegenden Dissertation handelt es sich um eine kumulative Arbeit.
Für die Arbeiten Möpert et al. und Jirikowski et al. war ich wesentlich daran beteiligt die verwendeten Methoden zu etablieren. Ich führte die Untersuchungen zum morphologischen und biochemischen Nachweis von CBG im Hypothalamus und der Hypophyse sowie die Kolokalisations-Experimente durch.
Für die Arbeit Lewis et al. etablierte ich einen Assay zur Konzentrationsbestimmung von CBG und führte die Plasmakonzentrationsanalysen für CBG durch.

Jena, den 10.03.2008 Benjamin Möpert